In vitro Green Globular Body Induction, Proliferation and Plantlet Regeneration of Adiantum reniforme var. sinense

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Research Article

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Abstract

The induction of green globular bodies (GGBs) represents an unique phenomenon in the tissue culture of pteridophytes. In this first attempt study GGBs were induced with the embryos of *Adiantum reniforme* var. *sinense*. The influences of basal salts, plant growth regulators, sugar concentrations, and gelling agents on the proliferation and differentiation of GGBs were examined. The highest induction rate of GGBs resulted from Murashige & Skoog (MS) medium supplemented with benzyl aminopurine (BA) (0.1 mg L^{-1}) and 1-naphthaleneacetic acid (NAA) (0.15 mg L^{-1}). The subsequent proliferation of GGBs required a higher concentration of NAA (10 mg L^{-1}) to have the highest fresh weight of 0.29 g. However, cytokinins, such as BA, kinetin (KT), and thidiazuron (TDZ), exhibited inhibitory effects on GGB proliferation. Hyponex was superior to MS medium in terms of GGBs proliferation and differentiation, which led to the highest fresh weight (1.72±0.63 g) and shoot number (7.25±1.89) after 60 days of cultivation. Among different sucrose concentration in media, GGBs cultured with 15 g L^{-1} sucrose had the highest fresh weight (1.01 g). Gelling agents also showed evident effects on the proliferation of GGBs, and the fresh weight of GGBs cultured in gelrite medium was approximately three times greater than that of GGBs cultured in agar medium. Distinct histological characteristics of embryos and GGBs were revealed by paraffin sections. All regenerates from GGBs survived in the greenhouse, with normal appearances, after acclimation in the medium of peat soil and pearlite (1:1).

Key Message

This study reported the first successful induction of GGBs from embryo of *Adiantum reniforme* var. *sinense*. The ideal media for in vitro culture of this fern was investigated.

Introduction

As an endemic pteridophyte with strong ornamental potential and herbal medicine value, *Adiantum reniforme* var. *sinense* is a perennial herbaceous fern, mainly distributed in the Three-Gorges area of the Yangtze River in China. Due to low reproductive ability through its spores spreading and difficulty in sporophyte cultivation, this fern is rarely grown commercially by horticulturists and gardeners. Moreover, wild population of *Adiantum reniforme* var. *sinense* has gradually become scarce due to sparse distribution, extractivism and destruction of natural habitats by the building of the Three Gorges Dam; thus, this fern is highly threatened and has been categorized as an endangered plant (Liu et al 2007).

GGBs are unique organized tissue emerging from explants in tissue culture of pteridophytes. They are usually induced with runner tips, leaves and leaf primordia of sporophytes as explants (Bertrand et al 1999;Higuchi et al 1987 Liao and Wu 2011 Li et al 2015 Yu et al 2017 Yu et al 2021). GGBs exhibit great potential to generate a large number of sporophytes due to the presence of several meristems inside a single GGB. Consequently, GGBs are widely applied in the production and species conservation of pteridophytes (Higuchi et al 1987 Fernández 2018). In *Adiantum reniforme* var. *sinense* GGBs have been induced with aseptic prothalli and whole sporophytes. The resulting GGBs induced with prothalli

differentiated into gametophytes, which is inconsistent with the purpose of production of sporophyte regenerates. Regarding induction with sterile whole sporophytes, GGBs were observed at least one month after tedious process for establishing aseptic sporophytes (Huang et al 2008 Wu 2009), which causing a need for a rapid protocol in tissue culture of *Adiantum reniforme* var. *sinense*.

In this study, GGBs were induced successfully with the embryos of prothalli and the effects of various components in the medium, including basal salts, sucrose concentrations, gelling agents, and plant growth regulators (PGRs) on GGB proliferation and differentiation were experimented. Also, the histological characteristics of embryos and GGBs were analyzed by microtome sections. Through induction of GGBs with embryos, as well as proliferation of GGBs, an in vitro reproduction system of *Adiantum reniforme* var. *sinense* is efficiently established. Moreover, we believe that, this is the first time that GGB from embryo has been observed in *Adiantum reniforme* var. *sinense*.

Materials And Methods

Plant material

The leaves of *Adiantum reniforme* var. *sinense*, were cut and washed with clean water, followed by sterilization with 75% alcohol and 0.6% sodium hypochlorite in sequence. These leaves were rinsed several times with sterile water, and further dried with filter paper. The sporangia of leaves were sliced and sown in a basal medium containing MS (Duchefa Biochemie, Haarlem, The Netherlands) with 30 g L^{-1} sucrose (Sigma Aldrich, St. Louis, MI, USA), 2 g L^{-1} Bacto-tryptone (BD, Biosciences, Franklin Lakes, NJ), and 4 g L^{-1} gelrite (Kelco, Rahway, NJ) with the pH adjusted to 5.8. Sterile gametophytes were formed, after spore germination within a month and small embryos were produced at the centers of several prothalli (Fig. 1a).

GGB induced with embryos

Sterile prothalli (approximately 0.2 cm in length) with embryos were cultured in MS medium with 30 g L^{-1} sucrose, 2 g L^{-1} Bacto-tryptone, and 4 g L^{-1} gelrite and adjusting the pH to 5.8. Different concentrations of BA (0, 0.1, 0.5, and 1 mg L^{-1}) and NAA (0, 0.15, 0.7, and 1.4 mg L^{-1}) were added for combinatorial experiments. Every treatment was repeated five times, each with the culturing of one explant. After 60 days, GGB number and weight produced by embryos under different PGRs were observed.

GGB proliferation and differentiation under different basal medium

The GGBs induced with the embryos of *Adiantum reniforme* var. *sinense* were used as explants, each with a weight of approximately 0.02 g. The basal media were prepared by supplementing MS medium with 30 g L^{-1} sucrose, 2 g L^{-1} Bacto-tryptone, and 4 g L^{-1} gelrite and adjusting the pH to 5.8. Then, the basal salts of the media were adjusted to 1/2 MS, full MS, and 3/2 MS (2.2, 4.4, and 6.6 g L^{-1}, respectively), as

well as 1/2 Hyponex (Hyponex Corporation, Marysville, OH), full Hyponex, and 3/2 Hyponex (1, 2, and 3 g L^{-1}) for the experiments. Every treatment was repeated four times, each with three explants. After 60 days, the fresh weight and shoot number protruding from GGBs were observed and recorded.

GGB proliferation and differentiation under different sucrose concentrations

The GGBs each with a weight of approximately 0.02 g were used as explants. The basal media were prepared by supplementing MS medium with 30 g L^{-1} sucrose, 2 g L^{-1} Bacto-tryptone, and 4 g L^{-1} gelrite and adjusting the pH to 5.8. The sucrose content of the basal medium was adjusted to 0, 15, 20, 30, and 45 g L^{-1} for the experiments. Every treatment was repeated four times, each with three explants. After 60 days, the fresh weight and shoot numbers protruding from GGBs were recorded.

GGB proliferation and differentiation under different gelling agents

The GGBs each with a weight of approximately 0.02 g were used as explants. The basal media were prepared by supplementing MS medium with 30 g L^{-1} sucrose, 2 g L^{-1} Bacto-tryptone, and 4 g L^{-1} gelrite and adjusting the pH to 5.8. The gelling agents in the basal medium were adjusted to the gelrite (3, 4, and 5 g L^{-1}) and agar (6, 7, and 8 g L^{-1}) (Hispanagar, Burgos, Spain) for the experiments. Every treatment was repeated four times, each with one explant. After 60 days, the fresh weight and shoot number protruding from GGBs were observed and recorded.

GGB proliferation and differentiation under different PGRs

The GGBs were used as explants, each with a weight of approximately 0.02 g. The basal media consisted of 3/2 Hyponex, 30 g L^{-1} sucrose, 2 g L^{-1} Bacto-tryptone, and 4 g L^{-1} gelrite, with the pH adjusted to 5.8. On this basis, different types and concentrations (1, 5, and 10 mg L^{-1}) of PGRs, including 2,4-D (2,4-Dichlorophenoxyacetic acid), BA, KT, NAA, and thidiazuron, were added. Basal medium without PGRs was used as the control treatment. Every treatment was repeated four times, each with one explant. After 60 days, the fresh weight and shoot number protruding from GGBs were observed and recorded.

Preparation of paraffin sections of embryos and GGBs

The embryos and GGBs of *Adiantum reniforme* var. *sinense* at different growth stages were cut into several pieces at appropriate sizes and placed in a mixed solution of F.P.A. (formalin 5%, propionic acid 5%, and ethyl alcohol 50–70%) for fixing, which was followed by 3–7 days of air extraction based on the tissue size. A series of dehydration procedures then performed with the mixture of tertiary butanol (t-butanol) and ethanol, and 0.5% safranin staining was finally performed. The dehydrated material was placed in an oven at 60–62 °C, thereby gradually increasing the concentration of paraffin in solution. After paraffin penetration, the block was cut into thin sections of 10–20 μm using a rotary microtome (Tominga, Taiwan). The paraffin sections were spread onto glass slides and air-dried for 2 days. Finally, the paraffin strips were dissolved in xylene and stained with 0.5% safranin and 0.1% fast green stain,

followed by mounting and air drying. Then, under an optical microscope, the histological examination for the embryos and GGBs at different stages was performed (Cason Jr 1974).

Statistical analysis

The data calculations and statistics for the experiments were performed with Microsoft Excel 2013 software, and the Statistic Analysis System (SAS) software package was applied to Duncan's multiple range test. The level of significant difference between treatments was defined at 5%.

Results

GGB induction with embryos

In the PGR-free basal medium, the sterilized spores of *Adiantum reniforme* var. *sinense* successfully generated and produced a large number of prothalli, which accidentally generated white embryos to grow into a complete sporophyte (Fig. 1a-b). When these white embryos were cultured in the medium supplemented with low-concentration of BA and NAA, yellow-green GGBs were protruded from the bases of these embryos within 14 days, which further developed into brown tissues covered by multilayered scales (Fig. 1c–d). As seen in Table 1, in the PGR-free medium, embryos directly grew into sporophytes instead of producing GGBs. By contrast, at low concentration of BA ad NAA, GGBs were successfully induced with embryos. In particular, the media supplemented with 0.1 mg L^{-1} BA and 0.15 mg L^{-1}NAA had the highest GGB induction rate and the maximum average fresh weight of 0.29 g. In addition, the growth of gametophytes was also promoted under the combined treatment with BA and NAA. Specifically, in the medium containing 1 mg L^{-1} BA, the gametophyte had the highest fresh weight of 0.67 g.

Table 1
Influence of BA and NAA on GGB induction from embryos of *Adiantum reniforme* var. *sinense* in vitro

PGR		Induction rate of GGBs	Average fresh weight of GGBs (g)	Average fresh weight of gametophyte (g)
BA (mg L^{-1})	NAA (mg L^{-1})			
0	0	0	0 d[z]	0.13 bcd
	0.15	0.6	0.03 bc	0.63 a
	0.7	0.4	0.02 bc	0.14 bcd
0.1	0	0.8	0.23 ab	0.08 cd
	0.15	1	0.29 a	0.16 bcd
	0.7	0.6	0.13 abc	0.03 d
0.5	0	0.2	0.08 abc	0.11 cd
	0.15	0.8	0.20 abc	0.17 bcd
	0.7	0.6	0.10 abc	0.04 cd
1	0	0.4	0.09 abc	0.67 a
	0.15	0.6	0.06 bc	0.49 ab
	0.7	0.8	0.10 abc	0.64 a

Mean value obtained from ten replicates. Data in the same column followed by the same letters are not significantly different at $p < 0.05$ using Duncan's multiple range test.

Influence of PGRs, salts, sucrose, and gelling agents on the GGB proliferation and differentiation

Figure 2 shows that the PGRs used in this experiment exerted evident influence on the proliferation of GGBs. In terms of auxin, the proliferation of GGBs was promoted by the increasing concentration of NAA, and the fresh weight of GGBs was highest in the case of 10 mg L^{-1} NAA. Though 2, 4-D played a similar role at 1 mg L^{-1}, the fresh weight of GGBs declined with the increase in concentration of 2–4, D. The growth of GGBs was inhibited when any type of cytokinin, such as BA, KT, or TDZ, was added to the medium, and they eventually caused the death of GGBs by high-concentration of BA and KT at 5 and 10 mg L^{-1} (Fig. 2).

As found in Fig. 3, Hyponex salts significantly outperformed MS salts in terms of both the proliferation of GGBs and number of emerging shoots. Regarding the proliferation of GGBs, the highest average fresh weight of GGBs (1.72 g) was obtained in full Hyponex medium (2 g L^{-1}), which was 3.8 times greater than the maximum fresh weight (0.45 g) obtained in full MS medium. The fresh weight of GGBs and

number of differentiated shoots evidently surpassed the promoting effects of PGRs, sucrose and gelling agents, which were the most influential factors of the medium in this study. Shoot differentiation was observed in full Hyponex and 3/2 Hyponex media within 60 days, and the highest average number of shoots was obtained in 3/2 Hyponex medium, wherein 7.25 shoots were produced. In contrast, no shoot differentiated from GGBs in MS medium (Fig. 3).

In the sucrose concentration test, the essential role of sucrose in the GGB proliferation and differentiation was revealed. As shown in Fig. 4, GGBs were subject to slow growth and the absence of shoot differentiation in the saccharide-free medium. By contrast, the fresh weight of GGBs in the medium containing 15 g L^{-1} of sucrose reached the highest average fresh weight of 1.01g. However, the fresh weight declined with the increasing concentration of sucrose in the medium. In terms of shoot differentiation, the concentration of sucrose had no obvious influence on the differentiation of GGBs (Fig. 4).

Regarding the influence of gelling agents on the GGB proliferation, gelrite distinctly outperformed agar. Specifically, after 60 days of cultivation, the average fresh weight of GGBs in medium containing 4 g L^{-1} gelrite was approximately twice as high as that in medium with agar (Fig. 5). In addition, GGBs failed to differentiate into shoots in medium with either gelrite or agar (data not shown).

Paraffin sections of embryos and GGBs of Adiantum reniforme **var.** sinense

As clearly observed in the paraffin sections of embryos of *Adiantum reniforme* var. *sinense*, a single meristem was clearly formed at the center of the embryo, surrounding by vascular tissues of primary leaves in a folded miniature sporophyte (Fig. 6a). Each embryo continues to grow into a complete sporophyte as in natural conditions.

Paraffin sections of the GGBs induced with embryos of *Adiantum reniforme* var. *sinense* revealed the multiple protrusion with densely arranged meristematic cell clusters in the peripheral of GGBs. For some meristems, vascular tissue of primary leaf was clearly observed, which intersected with each other as a continue circular through the entire GGB. Some meristems continued to differentiate, eventually producing multiple intact sporophytes at varying stages of development (Fig. 6b).

Discussion

GGBs are a special green granular tissue with several meristems and are induced by sporophyte tissues of pteridophytes in vitro. With multiple meristems, a single GGB can differentiate into several sporophytes, thus being considered a significant tool for mass production of pteridophytes. In 1987, Higuchi *et al.* first described and defined this unique structure on tissue culture of *Nephrolepis cordifolia Prsel*. Since then, GGBs have been induced with runner tips, leaves and primordia of leaf in several fern (Bertrand et al 1999;Higuchi et al 1987 Liao and Wu 2011 Li et al 2015 Yu et al 2017 Yu et al 2021). In this study, the induction of GGBs was achieved for the first time with embryos of *Adiantum reniforme* var. *sinense*. Based on the histological observation and subsequent shoot differentiation, it could be clearly

observed that multiple growing points differentiated into several individual sporophytes. These findings are different from those observed by Huang et al. (2008), in which GGBs were induced from the prothalli of *Adiantum reniforme* L. var. *sinense*, although gametophytes were ultimately formed. In the present experiments, the formation of a large number of GGBs was observed within 14 days, showing the highest induction rate and a rapid in vitro mass production of *Adiantum reniforme* var. *sinense*.

As found in this research, embryos can induce GGBs in medium containing low-concentration of BA (0.1 mg L^{-1}) and NAA (0.15 mg L^{-1}). However, the induction rate of GGBs was not raised by increasing the concentration of BA, which is different from the findings for GGB induction with higher concentrations of cytokinin BA (1 mg L^{-1}) in *Cibotium barometz* and *Pteris aspericaulis* var. *tricolor* (Yu et al 2017;Yu et al 2021). These results reveal that different plant hormones and concentrations are required to induce GGBs for different species of pteridophytes. To promote GGB proliferation, the combinations of auxin and cytokinin at various ratios were used in previous studies (Amaki and Kadokura 2009 Higuchi et al 1987 Yu et al 2017). However, it was found in this study that auxin of 2–4, D (1 mg L^{-1}) or NAA (10 mg L^{-1}) played the most evident role in promoting the GGB proliferation, while cytokinins (BA, KT, and TDZ) inhibited proliferation, and eventually leading to death. Our results in GGB induction and proliferation indicated that low concentration of NAA (0.15 mg L^{-1}) is needed for GGB induction, while high concentration of NAA (10 mg L^{-1}) is needed for subsequent GGB proliferation. Specifically, the optimum concentration of NAA for GGB proliferation is approximately 66 times higher than that for GGB induction.

As reported in previous studies, GGBs of *Cyathea lepifera* Copel. and *Microsorum musifolium* Copel. cultured in 1/4 MS medium produce more GGBs than those in full MS medium (Amaki and Toda 2010). However, our results indicated that the concentration of MS salts did not show a clear and significant difference in proliferation and differentiation of GGBs, while Hyponex medium markedly increased the fresh weight and shoot number of GGBs in this fern (Fig. 3). The promoting effect of Hyponex medium on the GGB proliferation and differentiation might be correlated to the relatively high content of trace elements and microelements in the medium. Despite the wide application of Hyponex in tissue culture of orchids (An et al 2021;Chen et al 2015 Ket et al 2004), there have been no reports on the use of the Hyponex medium in tissue culture of pteridophytes.

Sucrose serves as a carbon source, as well as a crucial component for maintaining osmotic potential in the medium. According to the observation, growth of GGBs was directly influenced by the presence of sucrose in the medium, however their fresh weights were reduced in medium containing excessively high concentrations (> 15 g L^{-1}) of sucrose (Fig. 4). Similar results have been obtained for other fern species *Diplazium nipponicum* (Amaki and Kadokura 2009).

The effect of the type and concentration of gelling agents on shoot regeneration has perviously been investigated (Debergh 1983;Kevers et al 1984 Pâques and Boxus 1987). The most widely known phenomenon is hyperhydricity, that is, low concentration of gelling agents causes a physiological malformation resulted in excessive hydration of seedlings. In the present experiments, the hyperhydricity was not occurred in low-concentration of gelrite and agar, while gelrite outperformed agar in promoting

the GGBs proliferation, with a difference of approximately twice the average fresh weight (Fig. 5). This result might be correlated with the fact that GGB growth is facilitated by the absence of residuals such as metal ions or phenolic compounds in gelrite and the superior light penetration due to the good transparency. Nonetheless, these two gelling agents displayed no effect on promoting differentiation of GGBs (data not shown).

From histological observation, embryos and GGBs of *Adiantum reniforme* var. *sinense* differed in their internal structures. The former only had one single meristem and hence could develop into a single sporophyte seedling. In contrast, the latter produced multiple protrusions of tightly arranged meristematic cells in the same annular vascular tissue. Similar results were obtained from paraffin sections of GGBs of *Nephrolepis cordifolia Prsel*, *Pteris aspericaulis* var. *tricolor*, *Asplenium nidus* L., and *Cibolium baromelz* (Higuchi et al 1987;Higuchi and Amaki 1989 Yu et al 2021 Yu et al 2017).

Conclusion

In conclusion, a rapid and highly efficient in vitro plant regeneration through GGB mediated protocol in *Adiantum reniforme* var. *sinense* was reported. Specifically, GGBs were successfully induced from embryos, which was the first study on embryos as the explant donor in this fern. The experiments revealed that the 100% induction rate could be obtained under BA (0.1 mg L^{-1}) and NAA (0.15 mg L^{-1}), and the medium containing 2 g L^{-1} Hyponex, 15 g L^{-1} sucrose, 4 g L^{-1} gelrite, and 10 m g L^{-1} NAA was most favorable for the succeeding proliferation of GGBs. However cytokinin such BA, KT and TDZ have adversely affect on GGB proliferation. In addition, 3/2 Hyponex PGR-free medium was optimal for shoot differentiation of GGBs. Moreover, paraffin sections were used to explore the difference between embryos and GGBs to achieve a better understanding of the development process of GGBs in *Adiantum reniforme* var. *sinense*.

Abbreviations

2,4-D: 2,4-Dichlorophenoxyacetic acid

BA: Benzyl aminopurine

GGB: Green globular bodies

KT: Kinetin

MS: Murashige & Skoog

NAA: 1-Naphthaleneacetic acid

ND: No surviving explants detected in the experimental group.

NS: No sporophyte detected.

PGR: plant growth regulator

TDZ: Thidiazuron

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Data Availability Statements

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

Ethic statements

All authors agree with the declarations that this submission follows ethical the responsibilities of authors as outlined in the Submission guidelines and Ethics & disclosures.

Authors Contribution

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Cheng-Wei Lin, Chia-Hung Shih and Chien-Yuan Kao. The first draft of the manuscript was written by Chien-Yuan Kao and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript

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Figures

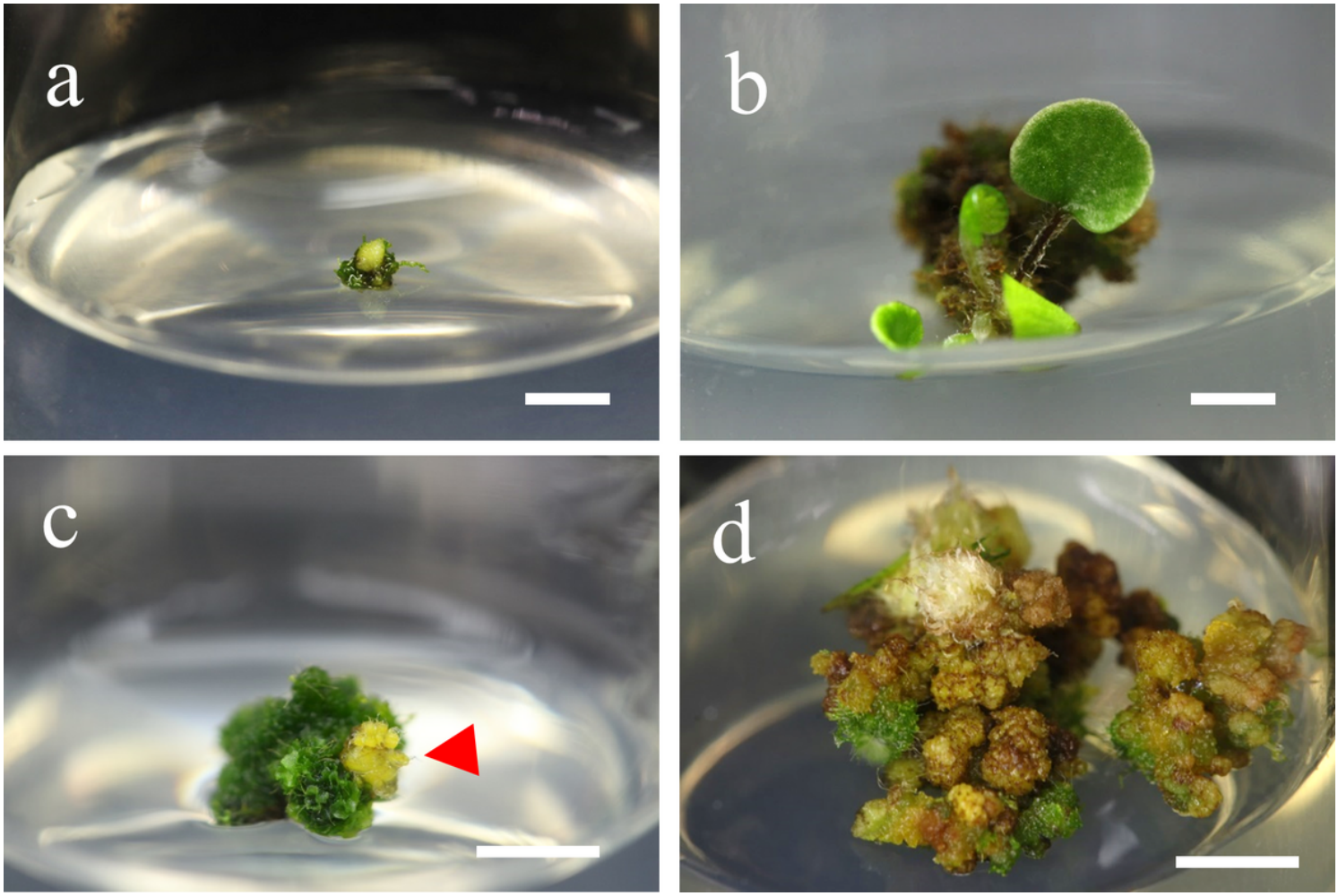

Figure 1

embryos, sporophytes produced by the differentiation of embryos, and GGBs induced with embryos (bar = 0.5 cm)

(a) White embryos produced at the center of green gametophytes

(b) Young sporophytes developed by embryos within 60 days in PGR-free basal medium

(c) GGBs formed within 14 days after embryos were cultured in medium containing PGRs

(d) Mature GGBs with brown tissues covered by multilayered scales

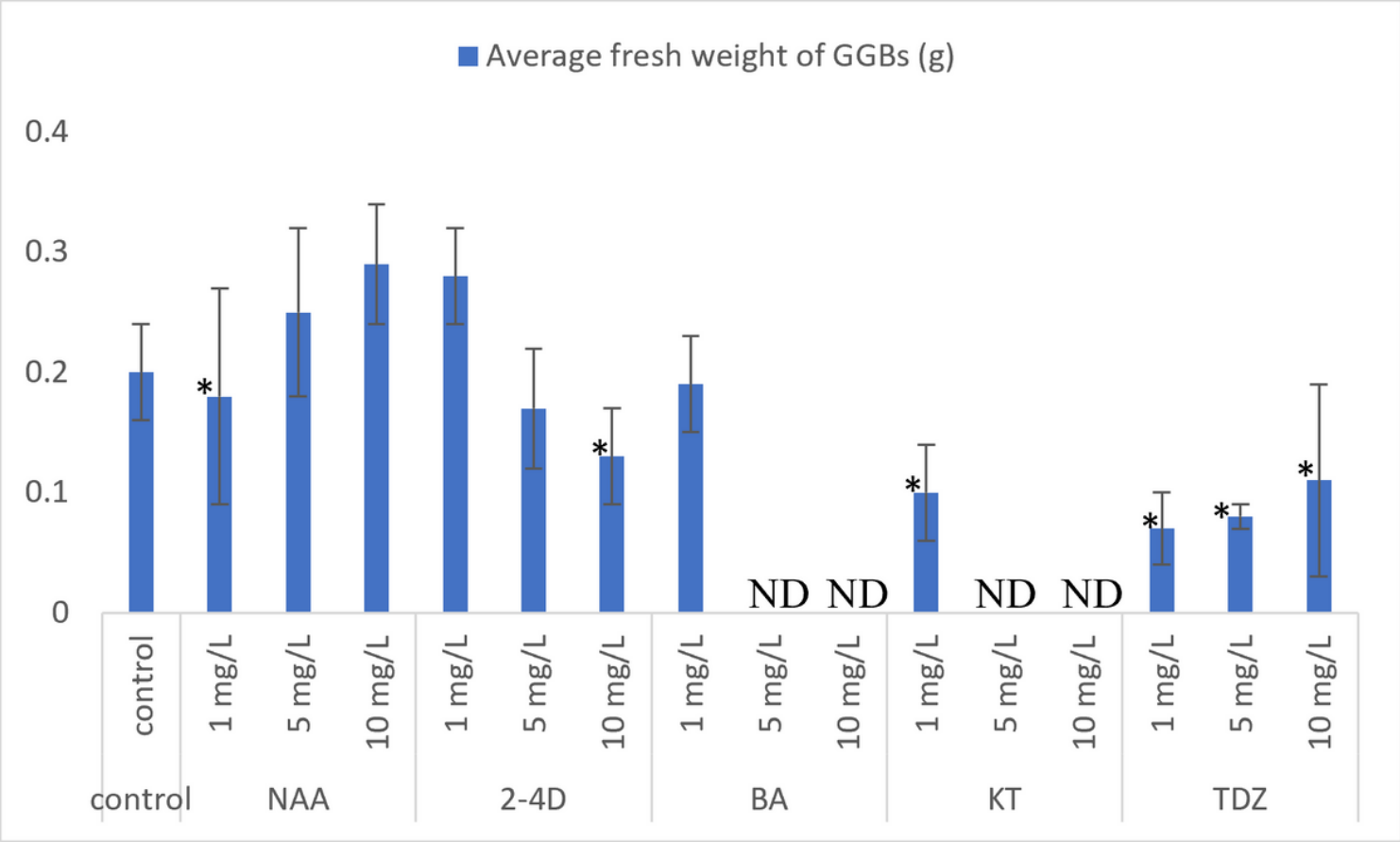

Figure 2

Influence of different PGRs on the proliferation of GGBs

Mean value obtained from four replicates using Duncan's multiple range test. ND: no surviving explants detected in the experimental group. *$p < 0.05$.

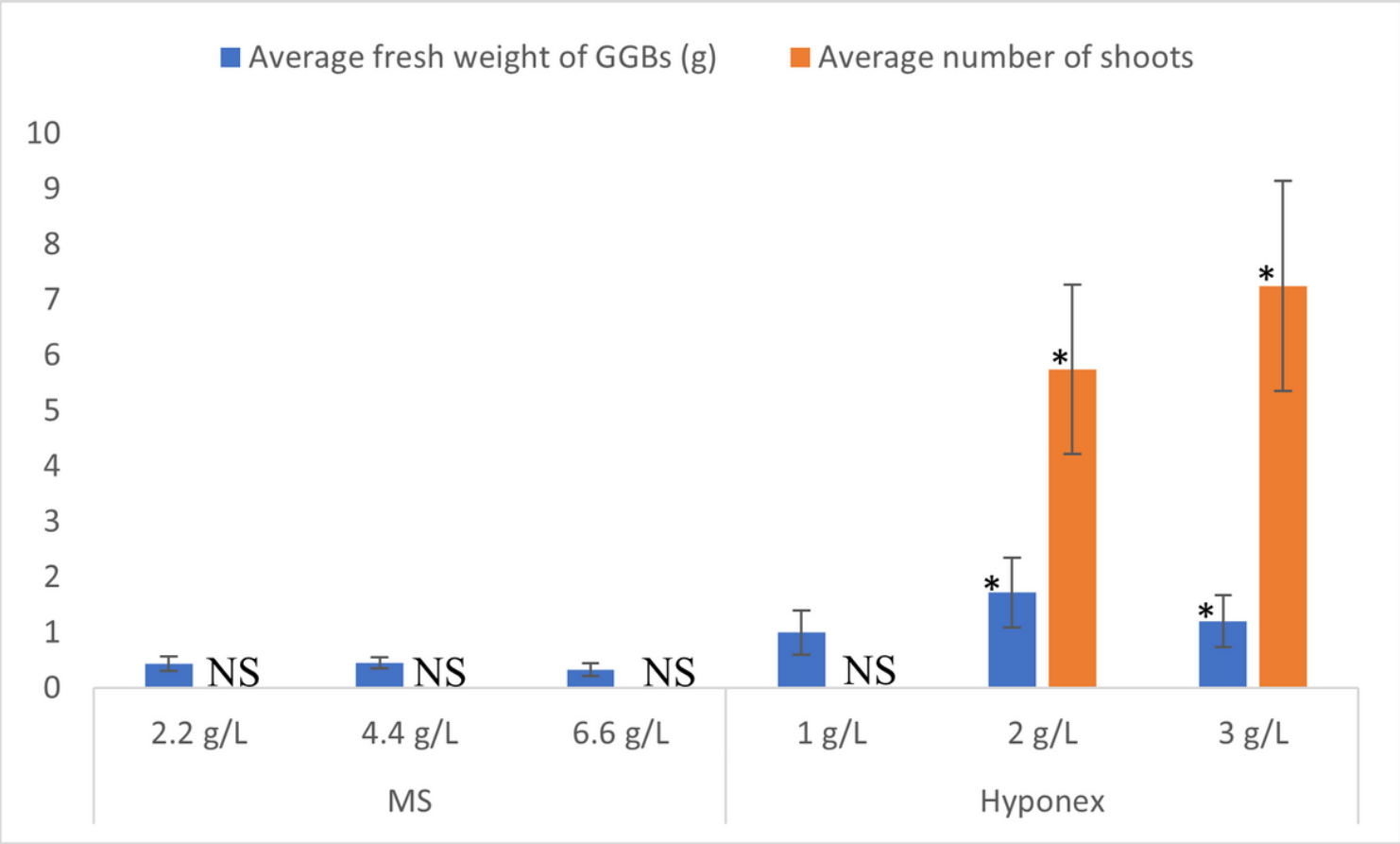

Figure 3

Influence of Hyponex and MS salts on proliferation and shoot differentiation of GGBs

Mean value obtained from four replicates using Duncan's multiple range test. NS: no sporophyte detected. $^{*}p < 0.05$.

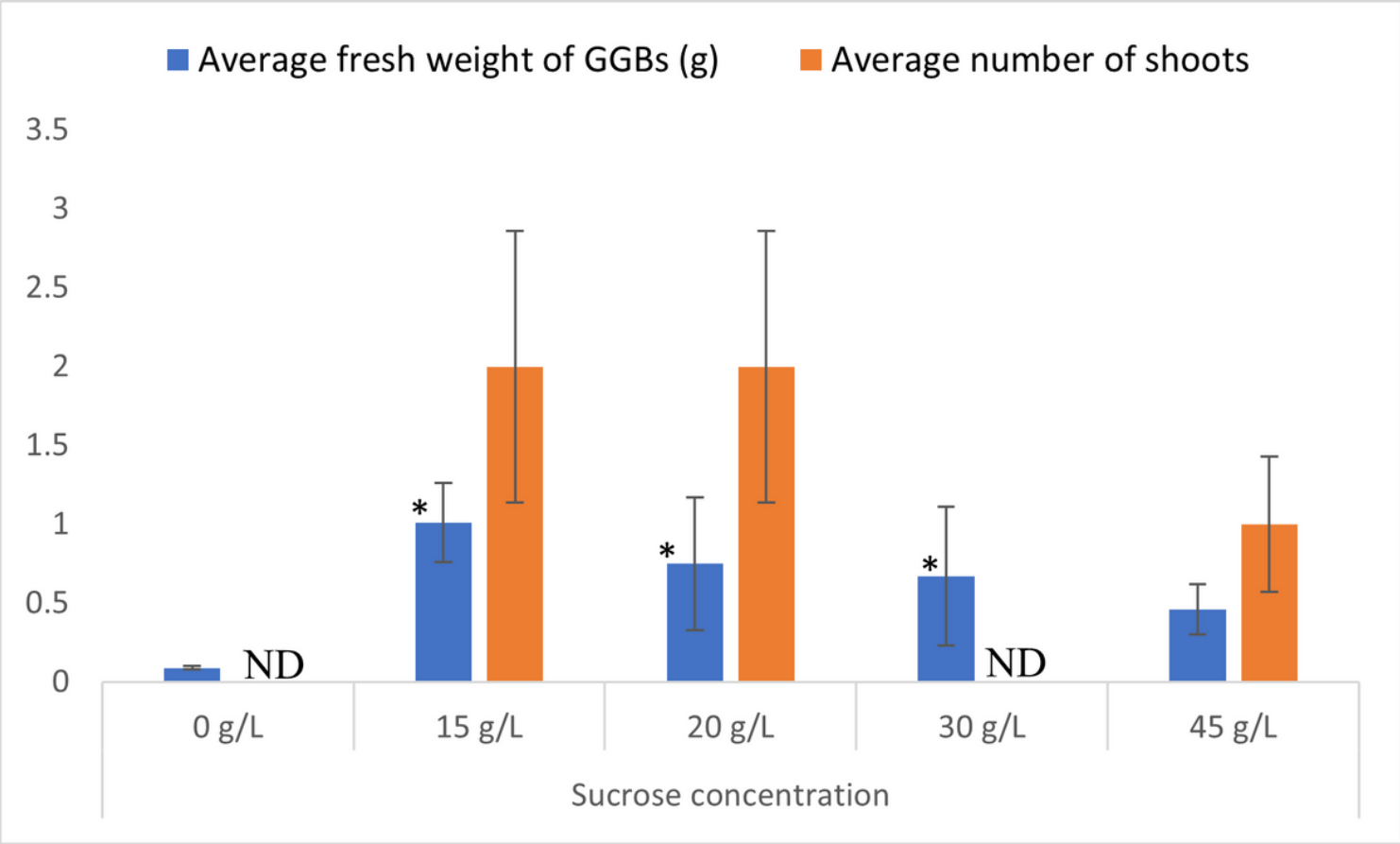

Figure 4

Influence of sucrose concentration on the proliferation and differentiation of GGBs

Mean value obtained from four replicates using Duncan's multiple range test. NS: no sporophyte detected. * $p < 0.05$.

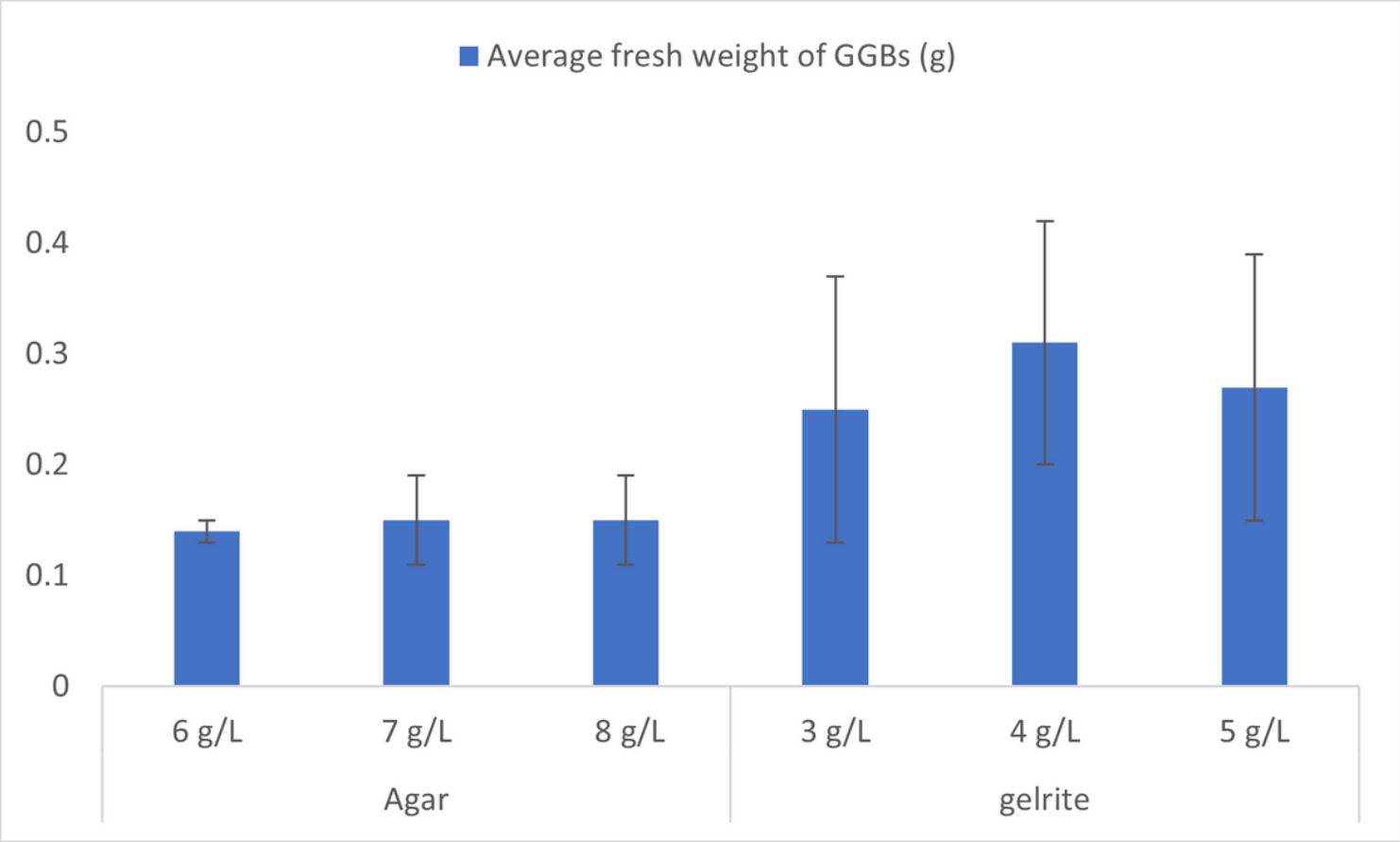

Figure 5

Influence of types and concentrations of gelling agents on the proliferation of GGBs

Mean value obtained from four replicates using Duncan's multiple range test. * $p < 0.05$.

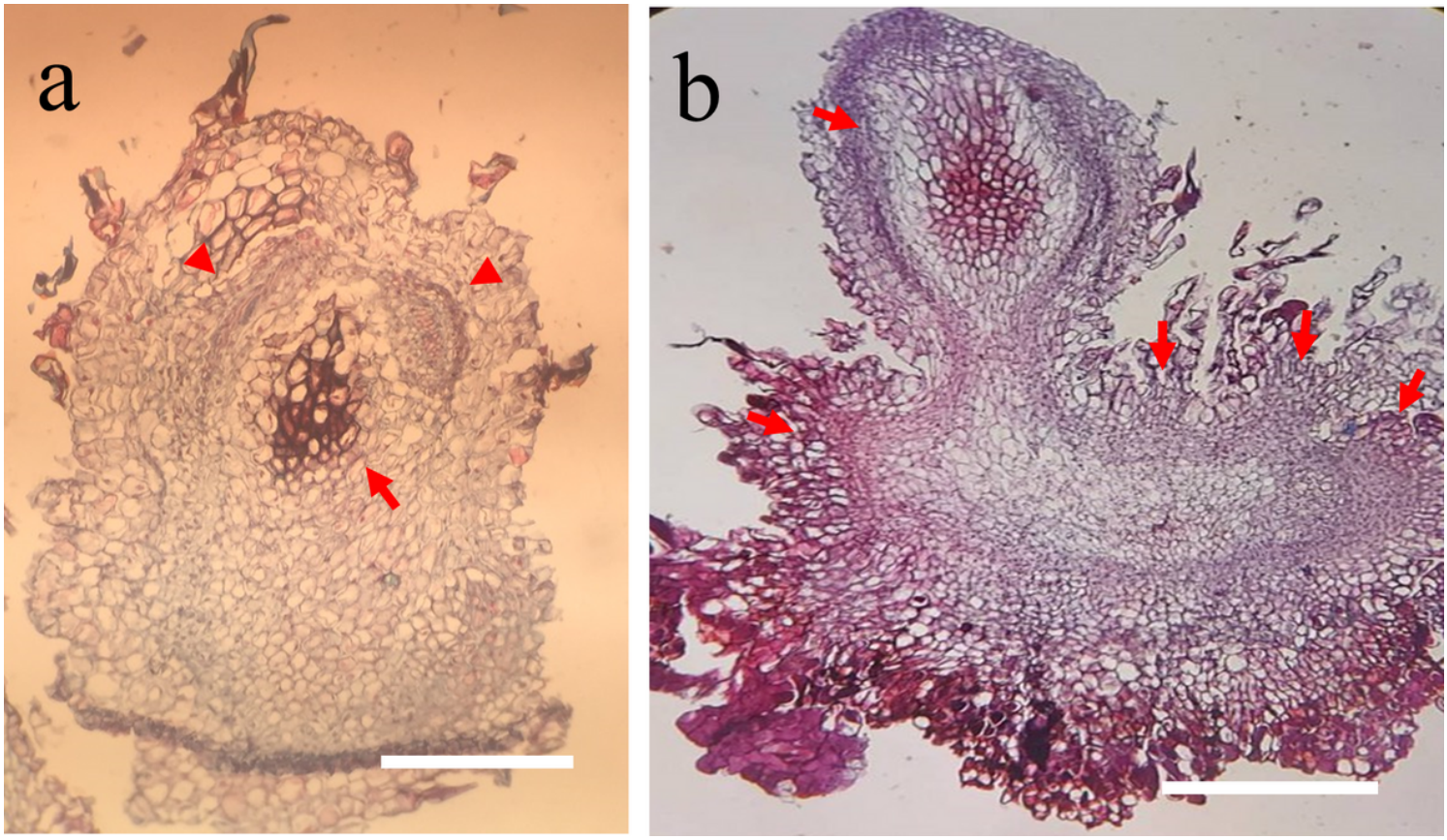

Figure 6

Morphology of paraffin sections of embryos and GGBs (bar = 0.1 cm)

(a) Section of an embryo showing a single meristem with densely arranged cells (arrow) and primary leaves (triangle).

(b) Section of a single GGB: a vascular ring is located near the surface and extends through the entire GGB, growing into several meristems (arrows) at different developmental stages and hence forming multiple sporophyte plants.