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Green Globular Body Induction and Plantlet Regeneration of Endangered Fern *Adiantum reniforme* var. *sinense*

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ABSTRACT

Background: *Adiantum reniforme* var. *sinense* is a pteridophyte with considerable ornamental importance and medicinal value. However, the wild population of *A. reniforme* var. *sinense* has gradually become scarce due to sparse distribution and human activities. Green Globular Bodies (GGBs) are specific tissue which use to propagate ferns *in vitro*. The study uses GGBs induced from sporophytic embryos to preserve and to proliferate this pteridophyte.

Methods: The present study was carried out during year 2018-2020 at National Ilan University. The developing sporophyte of *Adiantum reniforme* var. *sinense* were used to induce GGBs. The optimal medium for GGBs proliferation and differentiation in terms of basal salts, plant growth regulators, sugar concentrations and gelling agents was examined. The structure of GGBs and the embryos were analyzed by paraffin section as well.

Result: The result indicates the 100% GGBs induction rate from sporophytic embryos for the first time could be obtained under BA (0.1 mg L^{-1}) and NAA (0.15 mg L^{-1}). For subsequent proliferation of GGBs required a higher concentration of NAA (10 mg L^{-1}). Cytokinins such BA, KT and TDZ, inhibited GGBs proliferation. *In vitro* optimal amount of basal salts, plant growth regulators, sugar concentrations and gelling agents for GGB proliferation were obtained as 2 g L^{-1} Hyponex, 10 mg L^{-1} NAA, 15 g L^{-1} sucrose and 4 g L^{-1} gelrite, respectively. Paraffin sections revealed histological characteristics of the sporophytic embryo and GGB.

Key words: *Adiantum reniforme* var. *sinense*, Embryo, Hyponex, Plant growth regulators, Plant tissue culture.

INTRODUCTION

As an endemic pteridophyte with considerable ornamental importance and medicinal value, *Adiantum reniforme* var. *sinense* is a dwarfish unifoliate evergreen fern, mainly distributed in Asia and first reported its existence along both sides of the upstream Yangtze River in China (Lin, 1980). Due to low reproductive ability through its spores and lack of cultivation studies, this fern is rarely grown commercially worldwide. Moreover, the wild population of *A. 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, 2007).

GGBs are unique tissue emerging from explants in the tissue culture of pteridophytes. They are usually induced with runner tips, leaves and leaf primordia of sporophytes as explants *In vitro* (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 many sporophytes due to the presence of several meristems within a single GGB. Consequently, GGBs are widely applied in the production and species conservation of pteridophytes (Higuchi *et al*., 1987; Fernández, 2018). In *A. reniforme* var. *sinense*, the gametophytic GGBs have been induced with aseptic prothalli. The resulting GGBs differentiated into gametophytes, which is inconsistent with the purpose of sporophyte regeneration (Huang *et al*., 2008). Regarding sporophytic GGBs originating from embryos and their subsequent multiplication and differentiation, they haven't been recorded in previous studies of ferns.

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

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MATERIALS AND METHODS

The present investigation was carried out during year 2018-2020 at Orchid Biotech Lab, Department of Horticulture, National Ilan University, Yilan, Taiwan.

Plant material

The leaves with *Adiantum reniforme* var. *sinense* sporangia 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 dried with filter paper. The leaves were sliced and sown in a basal medium containing MS (Murashige and Skoog, 1962) (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 centre of several prothallium (Fig 1a).

GGB induced with embryos

Sterile prothallium (approximately 0.2 cm in length) with a single embryo was cultured in MS medium with 30 g L^{-1} sucrose, 2 g L^{-1} Bacto-tryptone and 4 g L^{-1} gelrite and adjusted 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 a single embryo under different PGRs were observed.

GGB proliferation under different basal medium

The GGBs induced with the *Adiantum reniforme* var. *sinense* embryo 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 twelve times. After 60 days, the total fresh weight of GGBs and protruding shoot number were observed and recorded separately.

GGB proliferation 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 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 twelve times. After 60 days, the total fresh weight of GGBs and protruding shoot numbers were recorded separately.

GGB proliferation 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 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. After 60 days, the total fresh weight of GGBs and protruding shoot number were observed and recorded separately.

GGB proliferation 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. 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. A basal medium without PGRs was used as the control. Every treatment was repeated five times. After 60 days, the total fresh weight of GGBs and protruding shoot number were observed and recorded separately.

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 were 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 the 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 of the embryo and GGBs at different stages was performed (Cason, 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 AND DISCUSSION

GGB induction with embryo

The sterilized spores of *A. reniforme* var. *sinense* germinated and produced many prothallia on the basal medium, which sporadically generated a yellowish embryo to grow into a

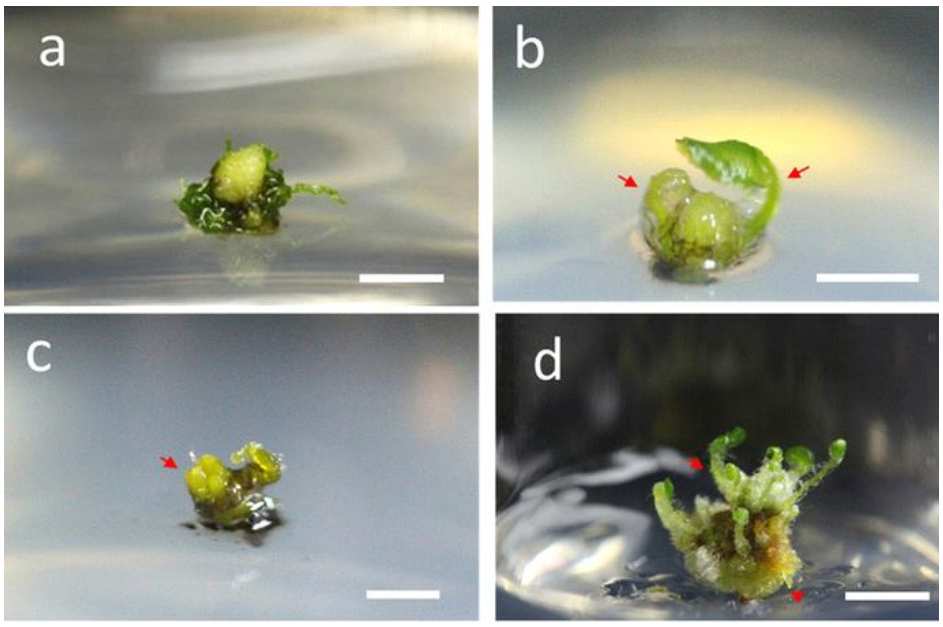
single sporophyte as in the natural life cycle of the fern (Fig 1a-b). When these embryos were isolated and cultured in the medium containing low amounts of BA or NAA, GGBs were induced from the embryos within 14 days, which subsequently differentiated into multiple ferns on the surface (Fig 1c-d). As seen in Table 1, there is no GGB formation from embryos cultured in the PGR-free medium. When media contained NAA and/or BA, embryos produced GGBs at every treatment with various efficiency. Specifically, the media supplemented with the lowest amounts of BA (0.1 mg L^{-1}) and NAA (0.15 mg L^{-1}) had the highest GGB induction from embryos with a maximum fresh weight of 0.29 g. By contrast, the growth of *A. reniforme* var. *sinense* gametophytes had the highest fresh weight of 0.67 g in the medium containing the highest BA (1 mg L^{-1}) in this experiment.

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

Fig 2 shows the PGRs exerted an evident influence on the proliferation of GGBs. In terms of auxin, the increasing amount of NAA promoted the proliferation of GGBs and the fresh weight of GGBs was highest in the case of 10 mg L^{-1} NAA. Though 2, 4 D played a promotive effect on proliferation at 1 mg L^{-1}, the fresh weight of GGBs declined with the increasing amount of 2, 4-D. However, the growth of GGBs was greatly inhibited by all kinds of cytokinin tested in this study. Eventually, it caused the death of GGBs by BA and KT greater than 5 mg L^{-1} (Fig 2).

As seen in Fig 3, Hyponex salts significantly outperformed MS salts in terms of GGB proliferation and the number of emerging shoots. The highest fresh weight of GGBs (1.72 g) was obtained in media containing 2 g L^{-1} Hyponex salts, which was 3.8 times greater than the maximum fresh weight (0.45 g) obtained in media containing 4.4 g L^{-1} MS salts. When GGBs were cultured on a medium containing 2 or 3 g L^{-1} Hyponex salts, all of them regenerated plantlets (Fig 3). The highest average number of shoots was obtained in 3 g L^{-1} Hyponex salts, wherein 7.25 shoots were produced. In contrast, no plantlet regenerated from GGBs in the MS medium (Fig 3).

The results of the experiment on the influence of sucrose concentrations are shown in Fig 4. The best result was obtained in the medium containing 15 g L^{-1} of sucrose, with the average fresh weight and number of shoots of GGBs reaching the highest of 1.01 g and 2 shoots respectively. In contrast, GGBs were subject to slow growth and no plantlets regeneration in the sucrose-free medium. However, both the fresh weight and the number of plantlets declined with the increasing concentration of sucrose in the medium (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



(a) White embryo produced at the center of green gametophytes; (b) Young sporophytes developed within 30 days in PGR-free basal medium (Arrows point to leaves of the developing sporophyte); (c) GGBs formed within 30 days after embryo was cultured in a medium containing PGRs (The arrow point GGBs); (d) Mature GGBs differentiate into sporophytes (Arrow indicates regenerated sporophyte and the triangle indicates undifferentiated GGBs).

Fig 1: Embryo, young sporophyte developed from embryo, GGBs induced with embryo and the differentiated ferns of GGBs (bar= 0.5 cm).

(Fig 5). In addition, GGBs failed to differentiate into shoots in medium with either gelrite or agar (data not shown).

Paraffin sections of an embryo and GGB of *Adiantum reniforme* var. *sinense*

Both embryo and GGB were sectioned and the internal structure was observed microscopically. As revealed in the histological examination of the embryo, a single meristem was observed in the centre of the embryo (Fig 6a). Each embryo continues to grow into a single fern as in natural conditions. However, the internal structure of the GGB revealed the existence of many meristems of different developing stages connected with the annular ring. With the development of meristems in GGB, many regenerated plantlets were formed on the surface of the swollen GGB (Fig 6b). The observation showed that GGB could be distinguished from the embryo.

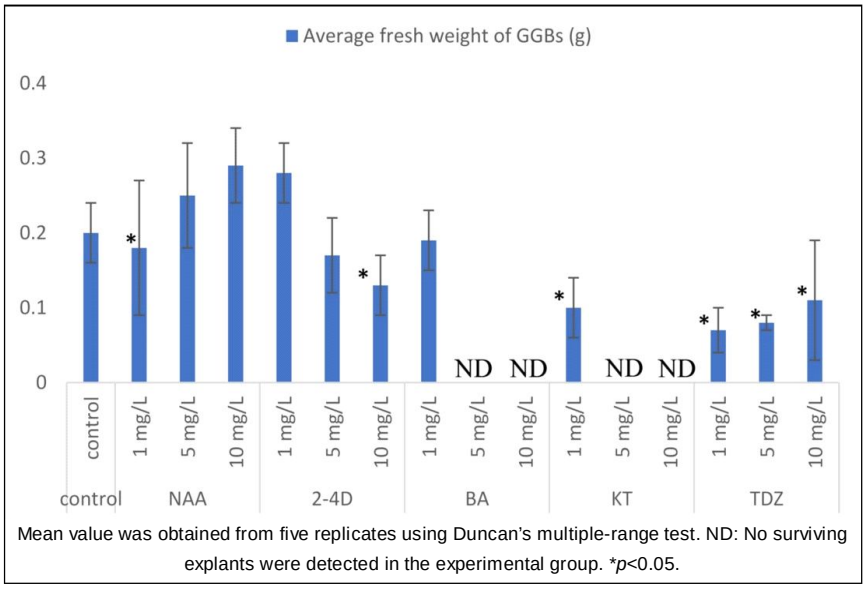
GGBs are unique structures induced by sporophyte tissue during *in vitro* culture. In 1987, Higuchi *et al.* first described GGBs in the tissue culture of *Nephrolepis cordifolia Prsel*. Since then, GGBs have been induced with runner tips, leaves and shoot primordia in several ferns (Higuchi *et al*., 1987; Liao and Wu, 2011; Li *et al.*, 2015; Yu *et al*., 2017). In this study, GGBs were induced for the first time with an *Adiantum reniforme* var. *sinense* embryo.

It was shown that all embryos produced GGBs easily in a medium containing low concentrations of BA (0.1 mg L^{-1}) and NAA (0.15 mg L^{-1}). The induction rate of GGBs was not increased by raising the amounts 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* (Li *et al.*, 2015; Yu *et al*., 2017). The combinations of auxin and cytokinin at various ratios were used successfully in previous studies to promote

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

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

Mean value obtained from five 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.



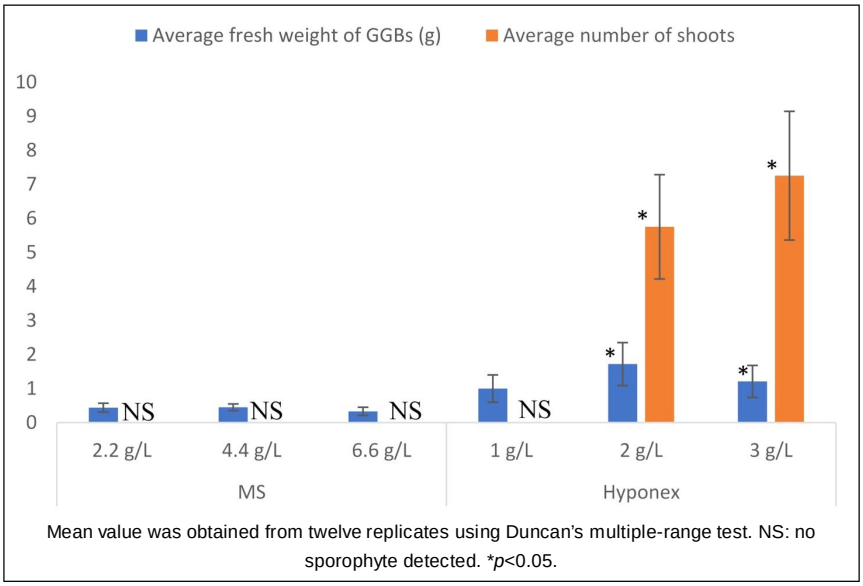
Mean value was obtained from five replicates using Duncan's multiple-range test. ND: No surviving explants were detected in the experimental group. *$p<0.05$.

Fig 2: Influence of different PGRs on the proliferation of GGBs.

GGB proliferation (Bertrand *et al*., 1999; Li *et al*., 2015; Amaki and Toda, 2010). However, in this study, only auxin of 2-4, D (1 mg L^{-1}) or NAA (10 mg L^{-1}) played the promotive role in the GGB proliferation, while cytokinins (BA, KT and TDZ) inhibited proliferation and eventually leading to the death of the GGBs. Specifically, a low amount of NAA (0.15 mg L^{-1}) is needed for GGB induction while a high amount of NAA (10 mg L^{-1}) is needed for subsequent proliferation. The optimum amount of NAA for GGB proliferation is approximately 66 times higher than that for GGB induction.

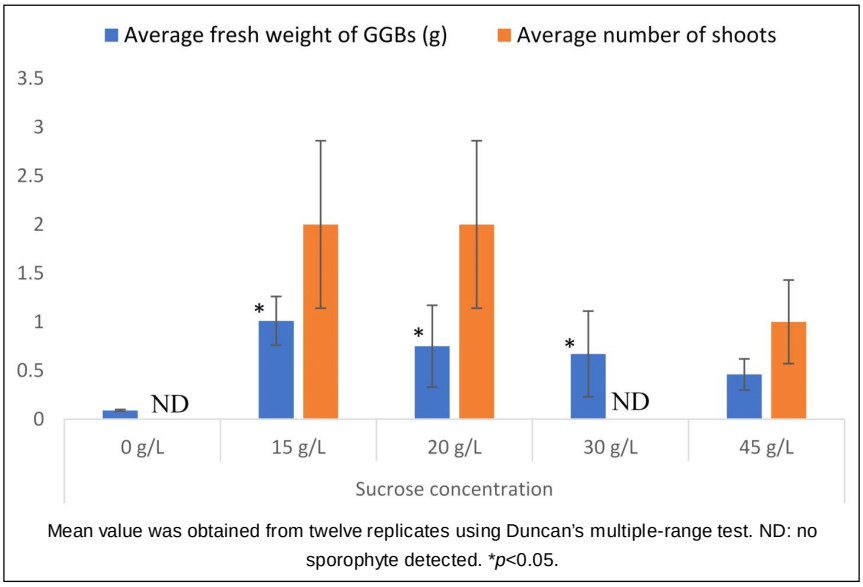
As reported in previous studies, most GGB related investigations were conducted in a medium containing the inorganic salts and vitamins of MS (Amaki and Toda, 2010). However, our results showed that GGBs were subjected to low proliferation and no plantlet regeneration in the MS medium, while the Hyponex medium markedly increased the fresh weight and plantlet number of GGBs. The promoting effects of Hyponex medium surpassed that of PGRs, sucrose and gelling agents, which were the most influential factors in this study. The promoting effect of the 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 the 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 the tissue culture of ferns.

Sucrose serves as a carbon source, as well as a crucial



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

Fig 3: Influence of Hyponex and MS salts on proliferation and shoot differentiation of GGBs.

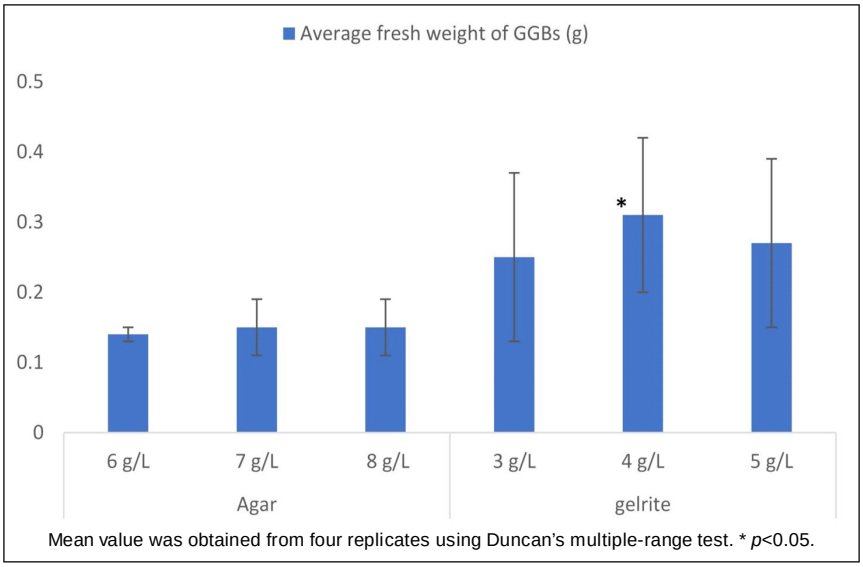


Mean value was obtained from twelve replicates using Duncan's multiple-range test. ND: no sporophyte detected. *p<0.05.

Fig 4: Influence of sucrose concentration on the proliferation and differentiation of GGBs.

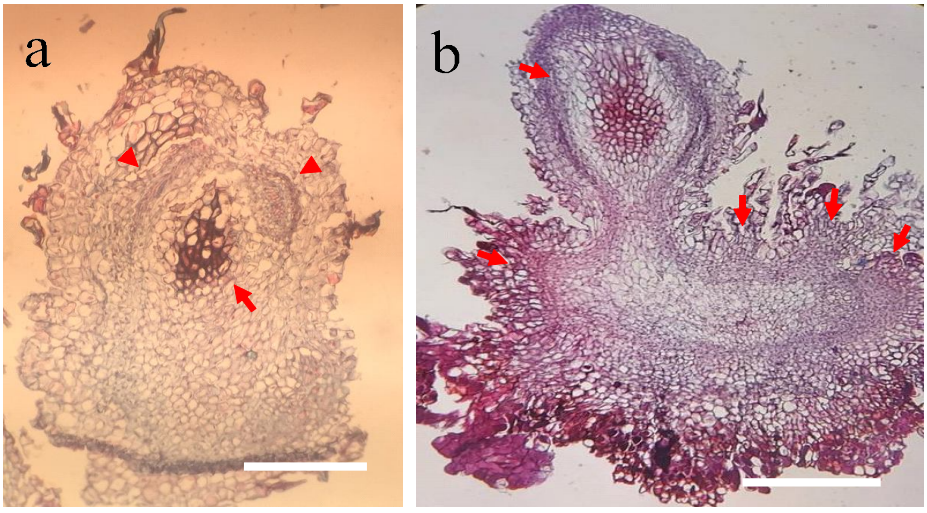
component for maintaining osmotic potential in the medium. In the experiments concerned with sucrose concentrations, the best result was obtained in a medium containing 15 g L^{-1} of sucrose. The GGB proliferation and plantlets regeneration was greatly promoted by the presence of sucrose in the medium, however, the promotive effect was reduced in a medium containing excessively high concentrations (> 15 g L^{-1}) of sucrose. Similar results have been obtained for other fern species (Amaki and Kadokura, 2009).

From histological observation, embryos and GGBs of *Adiantum reniforme* var. *sinense* differed in their internal structures. The embryo had only one meristem and subsequently differentiated into a single seedling. In contrast, the GGB had many meristems and multiple shoots are directly differentiated from these meristems and regenerated plantlets on the surface of the swollen GGB. It was shown that the vascular supply of shoots was found to be continuous with the vasculature of the GGB. The appearance of a vascular tissue connection between the regenerating structure and the explant is a major feature of organogenesis (Haensch, 2004; Wicart *et al.*,1984). Similar results were obtained from paraffin sections of GGBs of *Nephrolepis cordifolia Prsel*, *Pteris aspericaulis* var. *tricolor*, *Asplenium nidus* L. and *Cibolium baromelz*



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

Fig 5: Influence of types and concentrations of gelling agents on the proliferation of GGBs.



(a) Section of a developing embryo showing a single meristem with densely arranged cells (arrow) and primary leaves (triangle); (b) Section of a single GGB showing several meristems (arrows) and a vascular ring extends through the entire GGB.

Fig 6: Morphology of paraffin sections of embryos and GGBs (bar= 0.1 cm).

(Bertrand *et al*., 1999; Li *et al.*, 2015; Yu *et al*., 2017; Higuchi and Amaki, 1989).

CONCLUSION

This paper reported a highly efficient plant regeneration through GGB-mediated protocol in *Adiantum reniforme* var. *sinense*, which was the first study on embryos as the explant in fern. Specifically, the experiments revealed that a 100% induction rate could be obtained under BA (0.1 mg L^{-1}) and NAA (0.15 mg L^{-1}). For the maximum proliferation of GGBs the medium containing 2 g L^{-1} Hyponex, 15 g L^{-1} sucrose, 4 g L^{-1} gelrite and 10 mg L^{-1} NAA were most favourable. However, cytokinin such as BA, KT and TDZ have adverse effects on GGB proliferation. In addition, paraffin sections were performed to explore the difference between embryos and GGBs in *Adiantum reniforme* var. *sinense*.

Authors contribution

All authors contributed to the study's conception and design. Cheng-Wei Lin, Chia-Hung Shih and Chien-Yuan Kao performed material preparation, data collection and analysis. Chien-Yuan Kao wrote the first draft of the manuscript and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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This research received no external funding.

Data Availability Statement

Not applicable.

Ethics Statement

Our research complies with the Convention on Biological Diversity and the Convention on the Trade in Endangered Species of Wild Fauna and Flora. The plant material "*Adiantum reniforme* var. *sinense*" was bought from a local seller legally.

Conflict of Interest

The authors declare no conflict of interest.

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