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Morphological and Tissue Culture Studies of *Platycerium coronarium*, a Rare Ornamental Fern Species from Malaysia

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Abstract.—The genus *Platycerium* consists of about 18 species, commonly found in tropical and subtropical forests. Among the different species, *Platycerium coronarium*, *P. platylobium*, *P. ridleyi* and *P. wallichi* are found in Peninsular Malaysia, and *P. coronarium* is considered the most attractive ornamentally. *Platycerium coronarium* is an epiphytic fern, bears a gigantic morphology and is native to tropical areas of South America, Africa, Southeast Asia, Australia and New Guinea. *Platycerium coronarium* nests on the upper branches of the tallest trees in the forest. Due to having a uniquely-shaped fronds, they are famous for ornamental purposes, where they can be found in gardens, especially in tropical regions. Detailed morphological studies of this species are lacking. In the present work, data are reported aiming at defining both the macro- and micro-morphological characteristics of intact and *in vitro P. coronarium*. Data from scanning electron microscopy (SEM) revealed similar ultrastructures of both types of leaves, i.e., the presence of multicellular trichomes on both the abaxial and adaxial surfaces. Sunken stomata were also detected on the abaxial surface of the leaves. In addition, tissue culture studies were done to obtain an efficient regeneration system as well as to serve as an approach for conservation. Successful regeneration of sporophytes from gametophyte explants were observed in MS medium supplemented with 1.0–1.5 mg/l GA_3 and 30 g/l sucrose, at pH 5.8 under 16 hours light and 8 hours dark.

Key Words.—*Platycerium coronarium*, tissue culture, scanning electron microscopy (SEM), morphological studies, phytohormones

Platycerium (Polypodiaceae) consists of about 18 species, all of which are epiphytic and sometimes grow on rocks in tropical and subtropical forests. *Platycerium coronarium* (D. König ex O.F. Müll.) Desv., commonly known as staghorn fern, is an epiphyte that lives on large trees in most tropical forests of South-East Asia such as in Thailand, Myanmar, Philippines and Malaysia. The species is popular as an ornamental plant due to its uniquely shaped fronds. It is also valuable as a traditional medicinal plant to treat fever, irregular menstrual cycle and bile problems (Bidin, 1985). High demand for the limited number of species and forest destruction for development may lead to the extinction of this species (Porembski and Biedinger, 2001).

Few detailed morphological studies of *Platycerium coronarium* have been performed and limited tissue culture studies of *P. coronorium* had been carried out, such as those by Kwa et al. (1995a, 1995b, 1997). *In vitro* regeneration has

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TABLE 1. The effects of different concentrations of Naphthaleneacetic Acid (NAA) and 6-Benzylaminopurine (BAP) on the number of sporophyte leaves regenerated from gametophyte explants of *Platycerium coronarium.*

		Number of sporophyte leaves per explant		
NAA (mg/L)	BAP (mg/L)	Week 10	Week 20	Week 30
0.0	0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
0.1	0.1	3.25 ± 0.2	4.36 ± 0.2	5.25 ± 0.1
4.0	1.0	2.75 ± 0.5	4.65 ± 0.7	6.45 ± 0.3
0.1	1.0	0.00 ± 0.0	1.54 ± 0.6	2.32 ± 0.5
1.0	1.0	1.35 ± 0.6	3.65 ± 0.5	3.25 ± 0.4

Results: Mean ± S.E.

been recognized as a very useful technique for propagation and conservation of threatened plants. Gametophytic and sporophytic regeneration of scales of *Platycerium bifurcatum* (Cav.) C.Chr. has been reported (Ambro *et al.*, 1997) and regeneration of *P. coronarium* using leaf explants has also been achieved (Camloh *et al.*, 1994). To our knowledge, no successful protocol has been

FIG. 1. Habit of *Platycerium coronarium.*

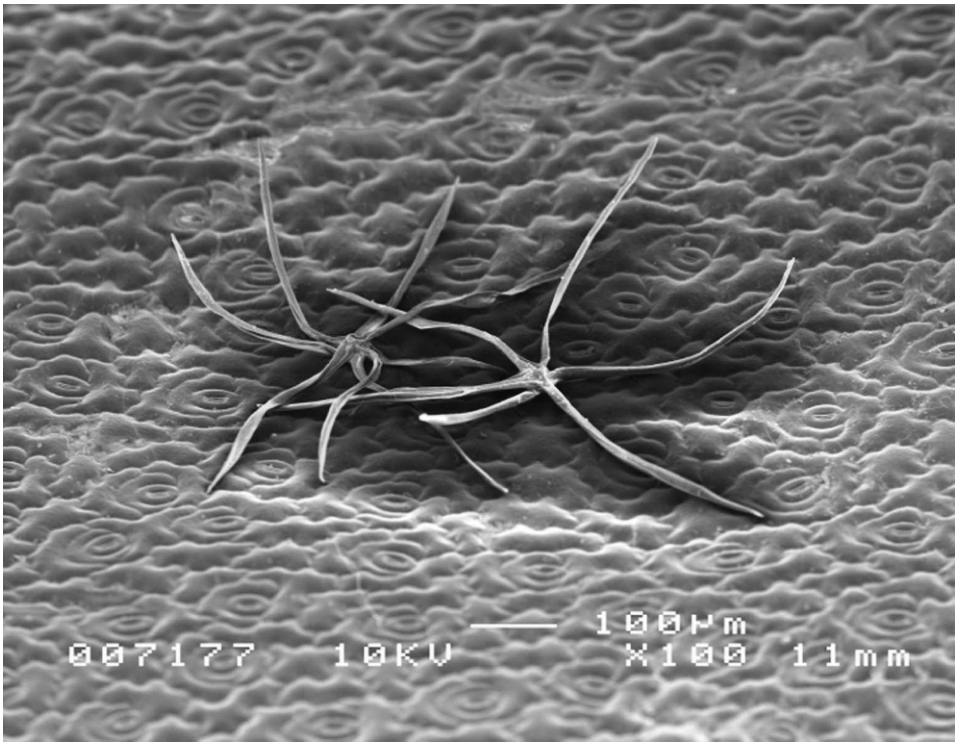

FIG. 2. Structure of trichome on the adaxial surface of *in vitro* leaf.

reported for shoot regeneration from *P. coronarium*, although recently different explants, such as spores (Aspiras, 2010), have been used.

In the present work, vegetative tissues (young sporophyte and gametophyte leaves) were used to regenerate *P. coronarium* utilizing the commonly used media (MS) (Murashige and Skoog, 1992), which has been used for regeneration of angiosperms (Taha and Tijan, 2002). The aim of the present investigation was to compare the macro- and micro-morphological characteristics of *P. coronarium* obtained both *in vivo* and *in vitro*. Also, an attempt was made to discover the most favorable sets of cultural and nutritional conditions for efficient *in vitro* regeneration, in order to establish an efficient regeneration system of *P. coronarium* and to ensure successful mass propagation of this species for conservation. Finally, the regenerated plants were compared with the intact plants to examine any variation that may have resulted from tissue culture protocols or due to different growth environments.

MATERIALS AND METHODS

Field studies and collection of specimens were conducted at Belum forest, Perak, Malaysia. For the tissue culture studies, sporophyte and gametophyte explants were used. Leaves sections (10 mm × 10 mm) from the young

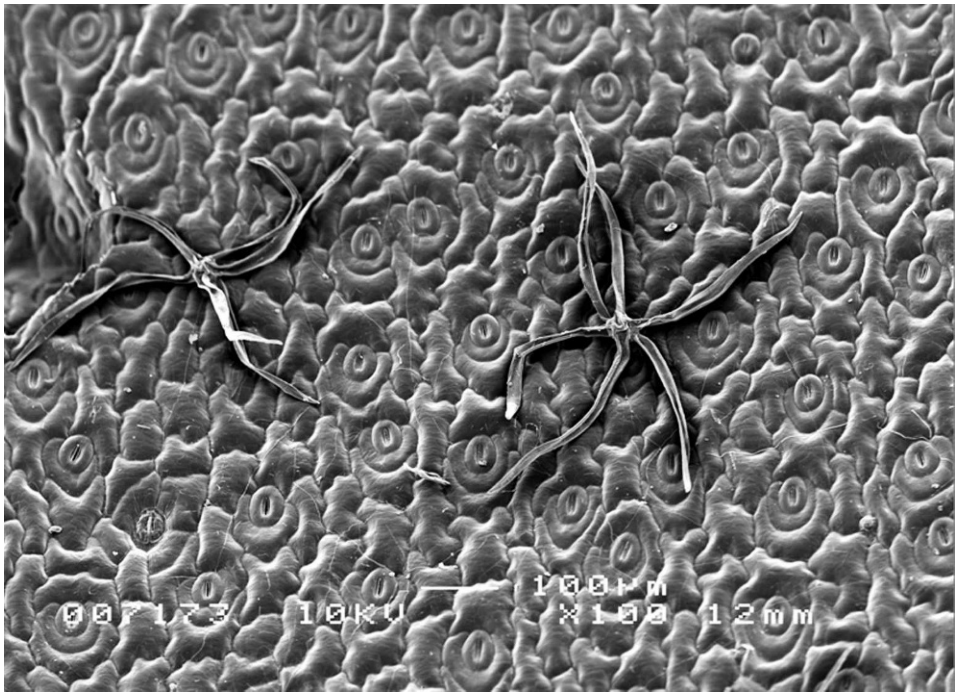

FIG. 3. Structure of trichome on the abaxial surface of intact leaf.

sporophyte plants and spores were sterilized using a series of sodium hypochlorite and alcohol, and explants were cultured following standard methods (Taha, 1993). The medium used throughout this study was MS (Murashige and Skoog, 1962) basal medium, supplemented with various hormones such as Naphthaleneacetic Acid (NAA), 6-Benzylaminopurine (BAP), and 2,4-dichlorophenoxyacetic acid (2,4-D), Thidiazuron (TDZ) and etc. at different concentrations and combinations (see Table 1). The cultures were maintained at 25 ± 1°C under 16 hours light and 8 hours dark for at least 30 weeks.

The morphological study was carried out using light and scanning electron microscopes to observe both macro and micro-morphology of intact and *in vitro* leaf samples of *P. coronarium*. Intact leaves were obtained from plants grown under natural environment, while *in vitro* leaves were obtained from plants grown under aseptic condition in tissue culture system. Samples used for both observations were the sporophyte leaves. For micromorphological studies, sections of clean leaves (3 mm × 3 mm) were immersed in glutaraldehyde and Sorencen phosphate buffer solution at 1:1 ratio for 1 hour. They were then rinsed with 50% Sorencen phosphate buffer and soaked overnight in 2% osmium tetroxide (OsO_4) at 4°C for 14 hours. The samples were washed with sterile distilled water after thawing at room temperature and subsequently soaked for 15 minutes each in 13 steps of distilled water and

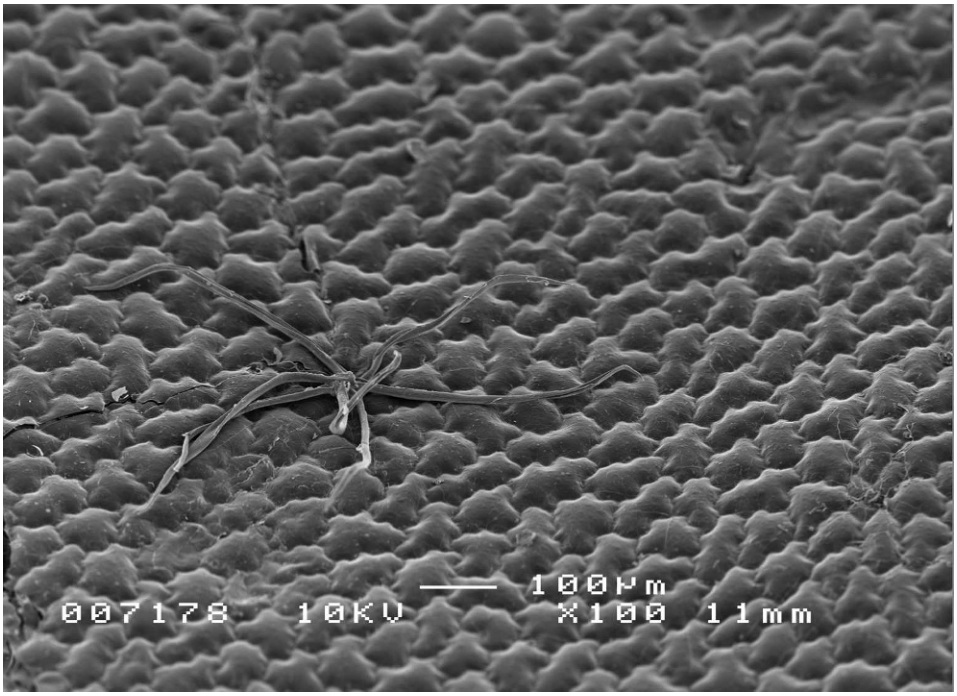

FIG. 4. Structure of trichome on the adaxial surface of intact leaf.

ascending concentration of diluted ethanol from 10% to 100%. This was followed by a hydration process using a series of 100% ethanol and acetone solutions in three different ratios (3:1, 1:1, 1:3). Samples were later soaked in 100% acetone for 20 minutes and this step was repeated four times.

The procedure continued with the drying process at critical point or Critical Point Drying (CPD) using a Polaron E 3000. Here, the samples were soaked in absolute acetone and inserted into the CPD E 3000 at low temperature (20°C). The sample was subjected to 'flushing' and impregnation using aqueous CO_2 and by controlling the temperature. Finally, the samples were kept in a drying box before the mounting occurred to view the samples under Scanning Electron Microscopes (SEM).

RESULTS AND DISCUSSION

In naturally growing *Platycerium coronarium* the fertile and sterile leaves differ morphologically. As observed in this study, fertile leaves are pendulous (up to 200 cm long), branch dichotomously and arise from the axil of the sterile leaf. Sterile leaves are arranged in the upright position, to trap nutrients from organic matter and can reach up to 60 cm long. Dead sterile leaves remain in their upright positions to trap water and humus from the environment (Fig. 1).

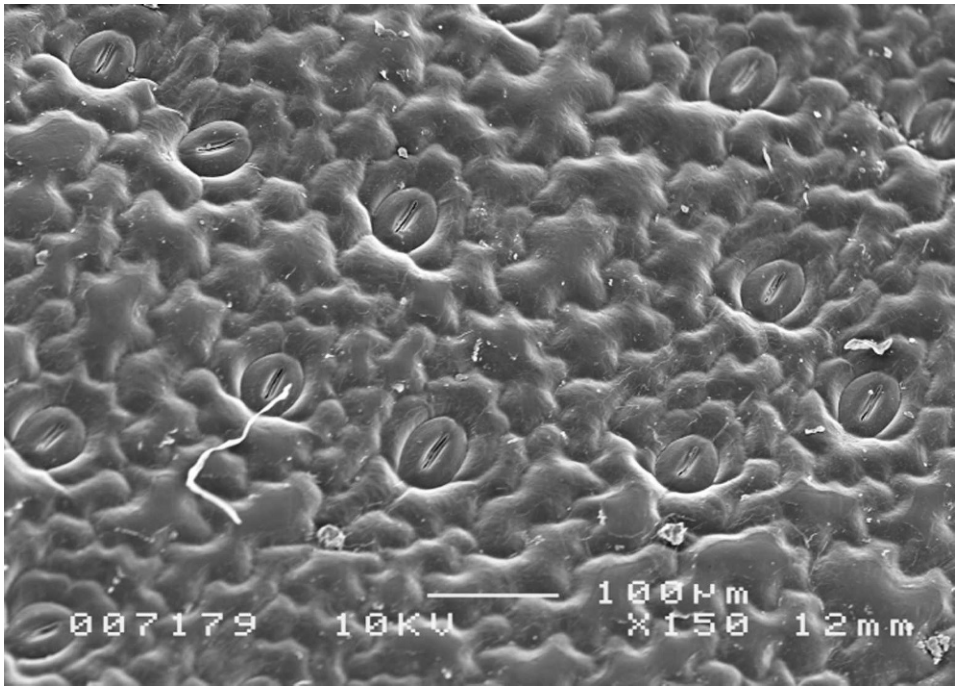

FIG. 5. Distribution of stomata on the abaxial surface of intact leaf.

Morphological observations were also made on sporophyte leaves of *Platycerium coronarium* regenerated in culture. Young sporophyte leaves have various shapes: round, cordate or lobed. The prothallus is thin, has a leaf-like structure and is photosynthetic and cordate. The size of the prothallus ranges from 0.05 to 0.3 mm. Rhizoids are present on the prothallus, functioning in the absorption of water for the gametophyte. Scanning electron microscopic observations of the prothallus showed that stomata and trichomes were absent. However, randomly distributed multicellular trichomes were observed on the abaxial surfaces of both *in vitro* and intact sporophyte leaves (Figs. 2–4). Anomocytic-type stomata, which are randomly distributed, were observed on both types of leaves, although more stomata were present on intact leaves compared to *in vitro* leaves (Figs. 5, 6, and 7). This may be due to the different environmental conditions of the species.

The most active part of the explant, being the sporophyte leaves, was identified when the explants were cultured on MS medium supplemented with 2 mg/l NAA and 1 mg/l BAP with a concentration of 30 g/l sucrose at pH 5.8 under 16 hours light and 8 hours dark compared to the other hormone combination. The formation of prothallus was observed after five weeks of culture, where the sizes ranged between 2.0–5.0 mm. MS medium supplemented with 0.1 mg/l NAA + 0.1 mg/l BAP induced production of 3.25 ± 0.2 sporophyte leaves after 10 weeks in culture using gametophyte explants (Table 1). MS medium supplemented with 1.0 mg/l NAA + 1.0 mg/l BAP

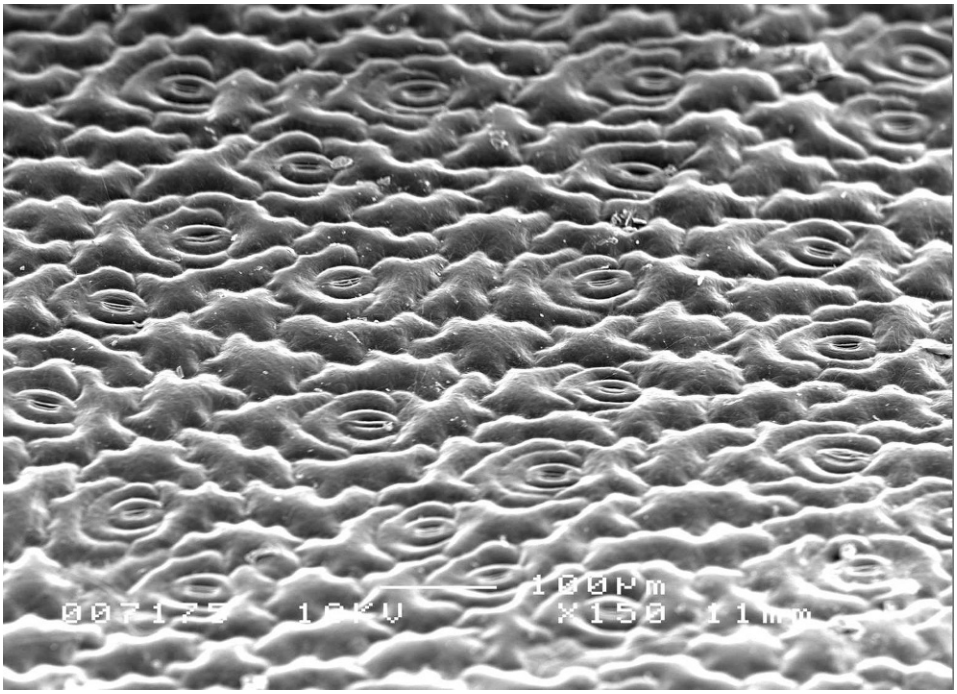

FIG. 6. Distribution of stomata on the abaxial surface of *in vitro* leaf.

resulted in production of the shoot-like structures after 10 weeks, with only 1.35 + 0.6 sporophyte leaves per explant. However, the number of leaves increased to 3.65 ± 0.5 and 3.25 ± 0.4 after 20 and 30 weeks, respectively. MS medium supplemented with 4.0 mg/l NAA and 1.0 mg/l BAP was the optimum media for regeneration of this shoot-like structure, which was observed after 20 to 30 weeks. The morphology of this shoot was globular and greenish in color. In the present study, the addition of 6-benzylaminopurine (BAP) alone to MS medium produced more shoots (1.35–6.45) but the formation of this shoot was slow compared to when MS medium without BAP was used.

TABLE 2. The effects of different concentrations of Gibberellic Acid (GA_3) on the number of sporophyte leaves regenerated from gametophyte explants of *Platycerium coronarium*.

	Number of sporophyte leaves per explant			
GA_3 (mg/L)	Week 5	Week 12	Week 23	Week 30
0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
0.1	0.00 ± 0.0	3.23 ± 0.2	9.25 ± 0.1	11.40 ± 0.4
1.0	0.00 ± 0.0	3.89 ± 0.7	20.45 ± 0.3	25.63 ± 0.6
1.5	0.00 ± 0.0	4.54 ± 0.6	18.32 ± 0.5	24.47 ± 0.8
2.0	1.23 ± 0.6	7.65 ± 0.5	10.25 ± 0.4	15.52 ± 0.2
3.0	0.00 ± 0.0	3.01 ± 0.5	8.75 ± 0.1	10.63 ± 0.3

Results: Mean ± S.E.

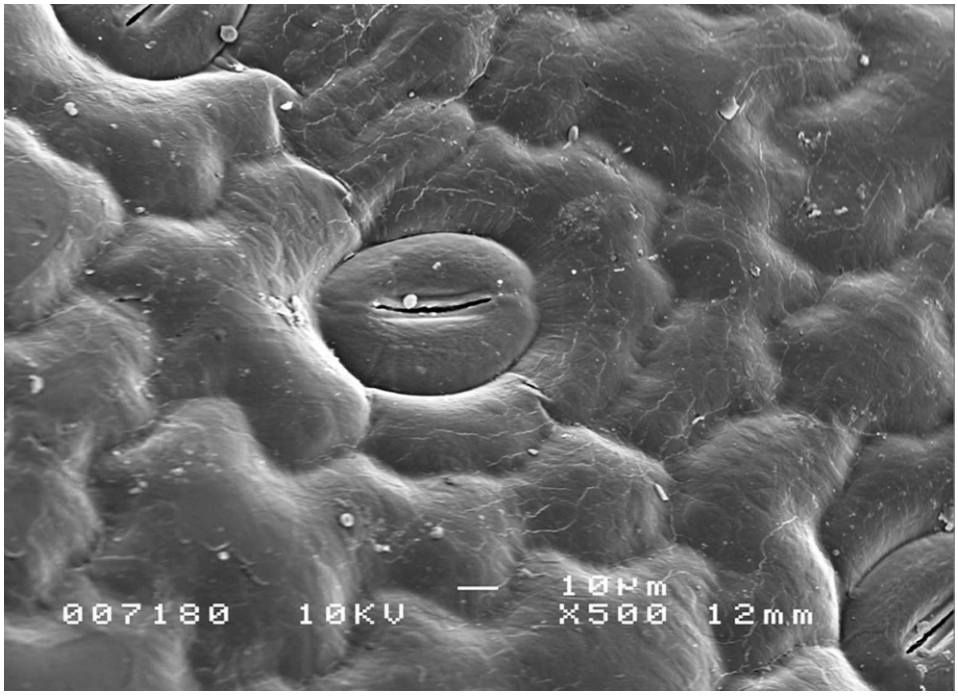

FIG. 7. Structure of stomata on the abaxial surface of intact leaf.

TABLE 3. The effects of various hormones : Indole-3-Acetic Acid (IAA), Kinetin, Thidiazuron (TDZ) and 2,4-Dichlorophenoxyacetic Acid (2,4-D) on the prothallus growth of *Platycerium coronarium.*

	Protallus growth (mm)		
Hormones	Week 1	Week 3	Week 6
0.1 IAA + 0.5 Kinetin	2.08 ± 0.5	2.38 ± 0.8	2.53 ± 0.3
0.5 IAA + 0.1 Kinetin	1.55 ± 0.1	2.09 ± 0.7	2.42 ± 0.2
3.0 IAA + 1.0 Kinetin	2.09 ± 0.8	2.56 ± 0.1	2.71 ± 0.1
1.0 IAA + 2.0 Kinetin	1.52 ± 0.4	2.05 ± 0.4	2.25 ± 0.5
0.1 2,4-D	1.26 ± 0.9	1.47 ± 0.9	0.00 ± 0.4
1.0 2,4-D	0.87 ± 0.7	0.98 ± 0.7	0.82 ± 0.6
1.5 2,4-D	1.08 ± 0.6	1.04 ± 0.6	0.00 ± 0.2
2.0 2,4-D	1.09 ± 0.3	1.09 ± 0.4	0.85 ± 0.1
3.0 2,4-D	1.06 ± 0.1	1.09 ± 0.7	0.96 ± 0.4
0.1 TDZ	1.35 ± 0.2	2.28 ± 0.9	2.58 ± 0.4
0.5 TDZ	1.08 ± 0.9	2.39 ± 0.8	2.59 ± 0.7
1.0 TDZ	1.59 ± 0.7	2.58 ± 0.5	2.77 ± 0.3
2.5 TDZ	1.0 ± 0.4	2.4 ± 0.4	2.63 ± 0.6

Results: Mean ± S.E.

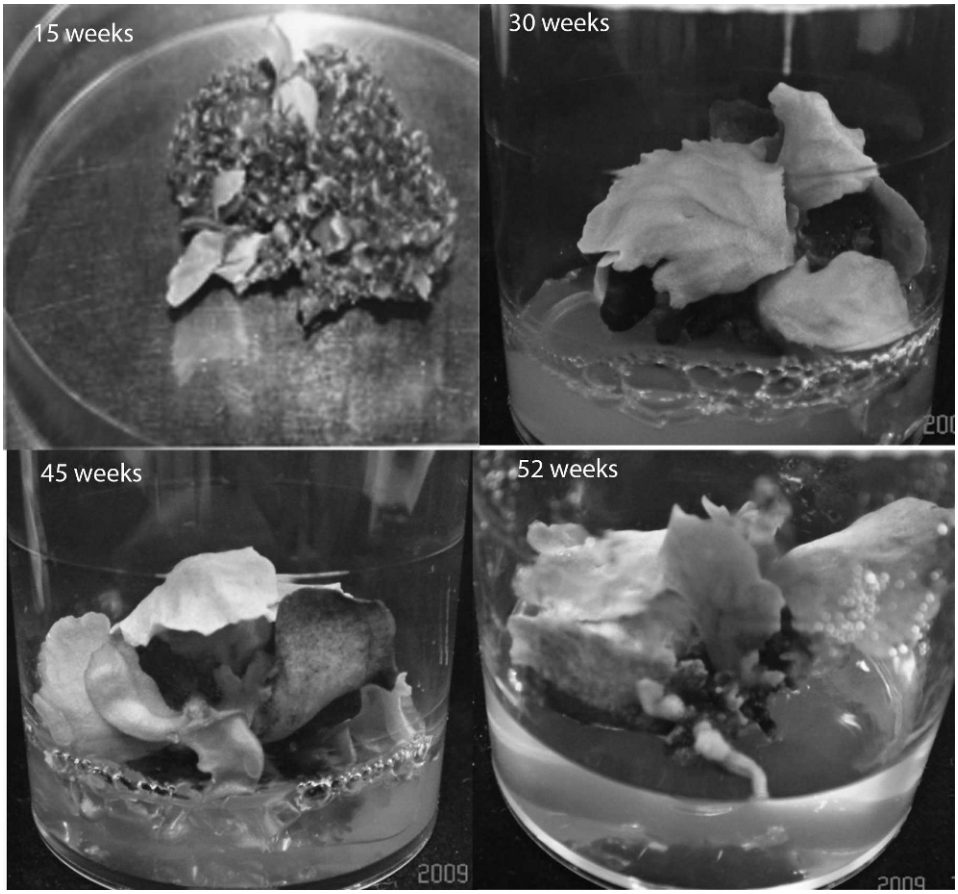

FIG. 8. Regeneration and propagation of *Platycerium coronarium* on MS medium supplemented with 1.0 mg/l Gibberellic Acid (GA_3) after 15, 30, 45 and 52 weeks.

Under favorable conditions, spores from gametophyte leaves will first develop into rhizoids and later mature to produce prothallus. In tissue culture systems, formation of rhizoid (root-like structure) from gametophyte leaves were obtained when the explants were cultured on MS medium supplemented with 0.1 mg/l NAA + 0.5 mg/l BAP and 1.5 mg/l NAA + 0.1 mg/l BAP after 10 weeks. However, the formation of the prothallus was very slow and no prothallus formed even after 20 weeks of culture when the above media and hormone combinations were utilized. Only after 30 weeks of culture, prothallus was formed. Thus, it can be concluded that formation of prothallus required a longer time compared to the rhizoids regeneration. In addition to NAA and BAP, Giberellic Acid (GA_3) was also tested using gametophytes as explants. The most responsive regeneration of sporophyte leaves was obtained in MS medium supplemented with 1.0–1.5 mg/l GA_3 (Table 2). Increasing concentrations of GA_3 (2.0–3.0 mg/l) generally lowered the number of

sporophyte leaves formed. GA_3 was more effective than NAA and BAP in the present study. Effects of Kinetin, IAA, 2,4-D and TDZ were also tested, and they were only effective in increasing the size of prothallus at a very low rates (Table 3). Figure 8 shows the development of *Platycerium coronarium* on MS medium supplemented with 1.0 mg/l GA_3 over the period of 15, 30, 45 to 52 weeks.

The present investigation indicates that regeneration and mass propagation of *Platycerium coronarium* is achievable and it can serve as an important tool for conservation of this rare and unique fern either *in vivo* after being acclimatized or in tissue culture systems as miniature plants. The present work differs from previous work, such as that of Aspiras (2010), where spores were used as explants, since we used vegetative tissues (gametophyte and sporophyte leaves) as explant sources. MS was utilized in the current work, which is normally used for seed plant regeneration. Hence, the current study has revealed that cultural and nutritional growth requirement of *Platycerium coronarium* can be met using this media. In the future, mass propagation of this fern species can be achieved using vegetative tissues and thus, reduces our dependence on spore germination for reproduction of this species.

Acknowledgments

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