The control of hormogonia induction/suppression by the alteration of scmucilage signaling molecules in Cycas plant in an endosymbiotic cyanobacterium

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Article

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Abstract

This study focuses on the role of mucilage signaling molecules secreted by the Cycad plant in the regulation of hormogonia induction/suppression in a novel endosymbiotic cyanobacterium (*Cyanocohniella cycadae sp. nov*.) isolated from the coralloid root of *Cycas circinalis*. Through a series of experiments using a combination of extraction, screening, acid-hydrolysis, derivatization, and GC-MS approaches, we sought to identify and characterize the mucilage signaling molecules secreted by *Cycas circinalis* and their role in the regulation of hormogonia induction/suppression in *Cyanocohniella cycadae*. Our results indicated that the mucilage components secreted in the coralloid root (CA) vary with the pre-coralloid root (PCA) of the same *Cycas* plant. Hence, we identified the presence of specific monosaccharides like arabinose (14.93 %), galactose (5.61 %), xylose (6.46 %), glucose (6.70 %), and altrose (4.41 %) in CA results in the suppression of hormogonia, whereas, glucose (29.86 %), fructose (18.86 %), talose (6.73 %), and lyxose (3.88 %) in PCA induces hormogonia development. Overall, this study provides new insight into the role of the alteration of mucilage signaling molecules. A shift between hexose and pentose in the pre-coralloid and coralloid root switches the induction and suppression of hormogonia in the cyanobacterium respectively. Further studies are needed to elucidate the mucilage biosynthetic pathways in the regulation of hormogonia induction/suppression in a variety of endosymbiotic cyanobacteria.

INTRODUCTION

The synthesis of a variety of specialised metabolites is one of the characteristics that distinguishes all land plants. The substances that the specialised metabolic pathways of plants manufacture serve a variety of purposes, ranging from the production of signaling molecules with nanomolar levels of abundance to superabundant structural polymers and compounds that protect against abiotic and biotic threats [1].

According to the endosymbiotic theory, non-photosynthetic eukaryotic host cells and photosynthetic cyanobacterial or algal endosymbionts ended up forming an endosymbiotic relationship that led to the evolution of photosynthetic eukaryotes [2]. The range of potential evolutionary outcomes for symbiotic relationships may be reflected in the different grades of symbioses, which can range from loose links to extremely specialised intracellular interactions. These radical evolutionary alterations and adaptations that came along with the development of plastids and mitochondria in eukaryotic cells. Age and the significant flexibility of plastids and mitochondria make it more difficult to research the processes that turn symbionts into organelles [3]. Existing symbiotic partnerships for nitrogen fixation exhibit varying degrees of adaptability and coevolution, illustrating several symbiont-host interaction phases.

The bacteria and archaea that fix atmospheric nitrogen and independent of another source of nitrogen are termed Diazotrophs. Irrespective of the family, genus, and species, the diazotrophic mechanism is widely spread among different taxonomic groups of bacteria and archaea [4]. The cyanobacteria are the pioneer colonizers of hot-desert soils [5], and cold-desert in Antarctica [6], cave inhabitants [7], symbiotic

associates of lichens [8], and are presumed as an ancient life form on Mars [9]. The cyanobacteria are the most successful oxygenic photoautotrophs that evolved 3.5 billion years ago, but the knowledge on their diversity is poorly known. Despite this, the polyphasic approach over the past decade had unveiled the discovery of many new genera and species.

Cyanobacteria are oxygenic, photosynthetic, Gram-negative bacteria, in which a small filamentous group generates a special type of cell called a heterocyst for nitrogen fixation and assimilation. In fact, plants including fern (Pteridophytes), liverworts (Bryophytes), cycad (Gymnosperms), and Gunnera (Angiosperms) let some diazotrophic cyanobacterium invade into a specific part of it for symbiotic association and benefited for nitrogen source [4]. The fern Azolla is the lone exception, where the cyanobiont is a vital component of the host during all phases of development and is passed on to the following generation. However, for the majority of endosymbioses, effective communication between the host and cyanobacterium is essential to the symbiont's successful invasion. Signal molecules play a prominent role during the early phases of invasion by the host and/or the symbiont. Subsequently requiring the host to release hormones that inhibit the growth of hormogonium in order to allow the cyanobacteria to form heterocysts so that nitrogen fixation can take place [10]. Heterocyst functions altruistically by supplying fixed nitrogen to nearby vegetative cells because they are terminally differentiated cells that are unable to divide [11].

The cycads are called “living fossils” and existed from the Mesozoic era (300 million years ago) and associated along with their counterpart, cyanobacteria through coevolution [12–15]. Cycads are unique gymnosperm plants that can fix atmospheric nitrogen (N_2) by symbiotically associated with cyanobacteria (phototrophic prokaryotes). With nine genera and 150 species, all of them are endemic to tropical and subtropical geographical locations [16,17]. The cyanobiont counterpart is allowed to colonize within a special type of dichotomously branched, Apo-geotropic roots with resemblance to corals are called coralloid roots [18]. These roots are found within 30 cm depth below ground level and may weigh up to 500 g of fresh weight [19,20]. Nevertheless, the mode of invasion of cyanobacteria into the coralloid roots of cycads is yet to be revealed. Despite some hypothesis claims that lenticels, papillose sheath [19], or aberrations in the dermal layer [21], and at the growing apex or intercalary region [20] were the possibilities favour cyanobacterial invasion.

The cyanobionts are the only tool for the plant host to fix atmospheric nitrogen and convert it into ammonia by the expression of the nitrogenase enzyme [22,23]. The nitrogenase enzyme expression in the heterocyst of cyanobiont is about 25–35% greater than that of the other vegetative cells [24,25]. The cross-section of the coralloid roots clearly showed that the cyanobionts colonized only inside the aerenchyma cells as a radial zone called an algal zone [26,27]. However, different *Nostoc* colonies may colonize inside the different individual coralloid roots of a single cycad plant [28].

The most common cyanobiont that colonizes inside the coralloid roots of *Cycas* plants was identified as *Nostoc cycadae* or *Anabaena cycadae*. Additionally, Calothrix sp. was found to colonize the coralloid root

of a cycad plant Encephalartos distributed in Africa [29]. Unfortunately, studies on the biodiversity, symbiotic relationship, and coevolution of the cyanobiont and cycads were still limited. Unlike other cyanobacteria, the *Nostoc* are differentiated into long vegetative filaments, short motile filaments termed Hormogonia, and heterocysts (N_2 fixing cells) [30]. The development of hormogonia is an important phenomenon in the life cycle of *Nostoc* stimulated by the hormogonia-inducing factors released by the nitrogen-starving host cycad plants to colonize the coralloid roots in the soil [30,31]. Nilsson et al. [32] reported that the signaling hormogonia-inducing factors were arabinose, glucose, and galactose synthesized as a slimy mucilage in the coralloid roots. However, there was a change in the signaling molecules after the colonization of *Nostoc* that induces the transformation of hormogonia into heterocyst (up to 30 to 40%) to induce nitrogen fixation [30]. Generally, simple sugars like sucrose, glucose, and fructose support heterotrophic mode in the cyanobiont to favour nitrogenase activity [30].

Whereas, in the *Gunnera* and *Nostoc* association, glucose and fructose were synthesized to sustain nitrogenase activity [33,34], and sucrose and dextrin enriches the *Nostoc* under dark or low light conditions [34]. Thus, it is evidently clear that during the course of coevolution, the host plant had evolved to alter and control the growth and differentiation of the cyanobacterium in the endosymbiosis associations [30]. In this mutualistic relationship, the host renders shelter and the endosymbiont provides various requirements of the host plant [35,36]. Most importantly, the arabinogalactan protein synthesized by the cyanobacteria [37] is utilized by the host plant in their growth and development [38], and signaling molecules in plant-microbe interactions [39,40].

Hormogonia are short filaments of cyanobiont that lack heterocyst but are motile enough for their dispersion [41] induced by the signaling molecules synthesized by the host plants [30, 42–44]. In the plant-cyanobiont symbiotic association, a set of regulatory genes in the host plant controls the expression of hormogonium-inducing factors that transform the cyanobiont colony into motile hormogonia [31,45] for their entry into the coralloid roots. Simultaneously, the signaling molecules are altered to suppress the hormogonia induction and enhance the development of heterocysts in the cyanobiont after colonization, in order to induce nitrogen fixation [42,45].

An acidic mucilaginous substance secreted in the cortex region[46] is the one that attracts the hormogonia filaments as in the case of *Gunnera*[32]. Concomitantly, mucilage-filled cavities were also reported in the liverworts and hornworts [47]. In parallel, the coralloid roots of cycads also synthesize a slimy mucilage at the root tip to trigger hormogonia differentiation in *Nostoc*[32,48,49].

Therefore, the aim of this study is to isolate a symbiotic cyanobacterial colony from the coralloid roots of the cycas plant and to identify the hormogonium-inducing factors synthesized by the plant host.

MATERIALS AND METHODS

Collection of coralloid roots of Cycad

The pre-coralloid and coralloid roots of the Cycas plant (*Cycas circinalis*) were collected from the Horticulture Farm, Madhavaram Botanical Garden, Madhavaram, Chennai, Tamil Nadu, India (Longitude: 13^{o}08’48.69” N, and Latitude: 80^{o}14’26.14” E). A sterile scalpel was used to cut the coralloid and pre-coralloid roots from the plant and brought safely to the algal culture laboratory, Department of Biotechnology, University of Madras, Guindy Campus, Chennai, in a sterile cover. After thorough washing in running tap water, thin cross-sections of the coralloid root were obtained by using a microtome and observed under a conventional microscope (Lawrence & Mayo, India) under 4 X, 40 X, and 100 X magnifications.

Scanning electron microscopy (SEM)

Thin sections of the coralloid root were heat-fixed (10^{o}C) on an aluminum sheet in order to remove moisture. Then the samples were coated with chromium using an ion sputter and were magnified under the Apreo S SEM (ThermoFisher Scientific).

Isolation of cyanobacterium

Fresh and thin coralloid root sections were subjected to surface sterilization with 10% Tween 20 for 5 min., rinsed, and again incubated in 4% hydrogen peroxide solution and 3% sodium hypochlorite solution for 3 min. each respectively. Finally, after incubating the root sections in 70% ethanol solution for ½ min., the thin sections were used as inoculum.

For isolation of the cyanobacterium, BG-11 broth and solidified culture medium were used under optimal light conditions, which were 10 hrs of light and 14 hrs of dark conditions at room temperature (28^{o}C to 30^{o}C). Serial dilution and frequent sub-culture strategies were implemented to yield a pure culture of the cyanobacterium. Then the pure cyanobacterial culture was further inoculated into a BG-11$_0$ nitrate-free medium to stimulate heterocyst and nitrogen fixation (**Table S1**). The isolated pure culture of the cyanobacterium was sub-cultured every 14 days and the purity of the culture was regularly examined under a microscope.

Morphological identification of cyanobacterium

The isolated pure culture of the cyanobacterium was morphologically similar to that of the *Nostoc* spp. [50]. However, there were unique features, such as the older cells being more differentiated in cell size, shape, and uneven distribution (constriction) compared to the young vegetative cells, and hence that was not confined to the *Nostoc*, it is strictly excluded from the *Nostoc in sensu stricto*.

Screening of hormogonium-inducing factor (HIF)

The fresh pre-coralloid and coralloid roots were washed thoroughly under running tap water to remove dirt and shade dried. About 0.5 g of the dried pre-coralloid and coralloid root was homogenized to a fine powder and extracted each with 5 mL of hexane, ethanol, and distilled water respectively. After incubation for 24 hrs at 4^{o}C, the hexane and ethanol extraction was slightly heated to 40^{o}C for 45 min. and centrifuged at 4000 rpm for 5 min. The solvent in the supernatant was evaporated and the residue was

dissolved in distilled water (**Table S2**). Whereas, the aqueous extraction was heated at 60^{o}C for 20 min. followed by the addition of twice the volume of 100% ethanol to precipitate the polysaccharides from the roots. The precipitated polysaccharide was collected by centrifugation at 3000 rpm for 5 min. The pellet obtained was dissolved in 2 mL of Milli-Q Water and utilized for the assay (**Table S2**).

About 12 different $BG11_0$ solidified plates were prepared for two different methods of screening including spread and streak for six different extracts respectively (**Fig. S1**), whereas different volumes of the extracts were given as 50 µL in disc 1, 100 µL in disc 2, and 200 µL in disc 3 respectively.

Characterization of HIF

Acid hydrolysis of HIF[51]

Each 10 mg of polysaccharide extracted from the coralloid and pre-coralloid roots was diluted in 1 mL of Milli-Q water in a test tube. About 0.5 mL of 10% HCl was added and subjected to boil for 1 hr at 80^{o}C.

Thin-layer chromatography (TLC) analysis of HIF[51]

The Silica gel plate 60 F_{254} (Merck, India) was used to perform TLC, in which the extracted and hydrolyzed polysaccharide of both CA and PCA was placed as a single spot on the TLC sheet separately. The mobile phase was the mixture of chloroform, acetic acid, and water in the ratio of 6:7:1. A 5% sulphuric acid prepared in ethanol was sprayed on the TLC plates and then the plate was heated at 50^{o}C on the hot plate to obtain dark spots.

Gas-chromatography and mass spectrometry analysis of HIF[51]

The acid-hydrolyzed polysaccharide samples (CA and PCA) were derivatized prior to GC-MS analysis. The hydrolyzed mixture was taken in a watch glass and dissolved in 0.5 mL of a mixture of solution constitutes of pyridine, hexamethyldisilazane, and trimethylchlorosilane in a ratio of 9:3:1 (v/v/v) respectively. Then the silylated samples were analyzed by GC-MS analysis using Agilent 7890B gas chromatography with an HP-5MS (5% phenyl methyl silox-) column of size 30m x 250 µm x 0.25 µm. The gas chromatograph was coupled with a 5877A mass spectrometry detector.

Conditions involved a sample injection volume of 1 µL with an injection temperature of 250^{o}C and detector temperature of 280^{o}C. The initial column temperature was 60^{o}C for a minute and was gradually elevated to 325^{o}C for the next 10 minutes. Helium was used as a carrier gas at a flow rate of 1 mL per minute with a split ratio of 1:1. The compounds were detected by the MS detector and identified by comparing them with the MS database from the National Institute of Standards and Technology MS2011 library. The obtained monosaccharide units of both the PCA and CA were tabulated and interpreted.

RESULTS

Collection of coralloid roots of Cycad

The collected coralloid root was obviously a coral-like structure with elongated internodes incorporated with a cyanobacterium, whereas, the pre-coralloid root is a newly arrived coralloid root from the cycas plant, in which the internodes were not much elongated and devoid of cyanobacterium incorporation (Fig. 1).

Isolation of cyanobacterium

Figure 2**(A & B)** shows the cross-sectional view of the coralloid root of the cycas plant. In which, the aerenchyma cells were arranged as a radial ring invaded by the symbiotic 3.2. cyanobacterium. The same was isolated from the coralloid root collected from the *Cycas circinalis* and pure cultures were maintained. Figure 2**(C & D)** illustrates the scanning electron microscope images of the cross-section of the coralloid root of the cycas plant. It is obviously visible that the empty aerenchyma cells were invaded by the cyanobacterium, and were unfortunately removed during the process of specimen preparation for SEM analysis.

Morphological identification of cyanobacterium

Initially, the cyanobacterium isolated from the coralloid root of *Cycas circinalis* was identified as *Nostoc* sp. (**Fig. S2**) based on morphological features similar to that of the *Nostoc*. But, upon frequent microscopic observations, different morphological features were observed and photographed. Based on that, the isolated cyanobacterial strain was not *Nostoc* and thus excluded from the *Nostoc in sensu stricto*.

Three different characteristic features were seen in the polymorphic life cycle of the cyanobacterium, which includes the *Pseudoanabaena*-like stage, in this stage, the hormogonia and young filaments were straight and not constricted. In addition, the terminal cells were not wider than the intercalary cells. The average length and width of the terminal cell were 4.59 µm and 4.16 µm respectively, whereas, the same for the intercalary cells were 4.23 µm and 4.83 µm. The second stage is the *Nostoc*-like stage, the size and shape of the cell were not even and slightly constricted. The average length and width of cells in this stage were 4.5 µm and 6.5 respectively. The *Chlorogloeopsis*-like stage constitutes irregular-sized mature cells, which were highly constricted with multiple branching and witnessing cell division in two planes. The *chlorogloeopsis*-stage leads to akinetes and their germination in the life cycle (Figs. 3 & 4; **Fig. S3)**.

These unique features in the life cycle of the cyanobacterium isolated from the coralloid root of *Cycas circinalis* were found similar to that of the genus *Cyanocohniella*. It is the newly classified genus separated from *Nostoc in sensu stricto* in the year 2014 [52]. However, *Cyanocohniella calida*, and *C. crotaloides* were the two type species published so far [52,53]. *Cyanocohniella calida* was isolated from the hot-water thermal springs of Karlovy Vary (Carlsbad), Czech Republic at 47^{o}C to 55^{o}C of water temperature in the year 1996 and named *Mastigocladus laminosus*[54], again re-isolated in 2012 and named *Cyanocohniella calida gen. et sp. nov.* in 2014. Contrastingly, Jung in the year 2018 isolated a

cyanobacterium with similar kind of features from the beach mat of Barrier Island, Schiermonnikoog, Netherlands at a warmer temperature of 8^{o}C and named it *Cyanocohniella crotaloides sp. nov.*[53].

Simultaneously, in this study, a symbiotic cyanobacterium with the same morphological features was isolated from the coralloid roots of *Cycas circinalis* (28^{o}C to 30^{o}C) from Chennai, India. Therefore, the new species of cyanobacterium was named **Cyanocohniella cycadae sp. nov.**

Habitat

Symbiotic association inside the coralloid roots of *Cycas circinalis.*

Etymology

The species named *'cycadae'* as the cyanobacterium isolated from the coralloid root of *Cycas* plant.

Screening of hormogonium-inducing factor (HIF)

To determine the hormogonium-inducing factor, three different extracts of both coralloid and pre-coralloid roots were prepared and tested to induce hormogonia in the newly isolated cyanobacterium *Cyanocohniella cycadae*. **Figure S4** represents that the three different extracts aqueous (CA), ethanol (CE), and hexane (CH) at different gradient concentrations from discs 1 to 3 in the spread plate showing no dispersion of cyanobacterium towards the disc. However, the dispersion was observed only in the aqueous extract (CA) of the coralloid root in streak plate method (**Fig. S5**). Whereas, in the case of pre-coralloid extracts, the dispersion was seen only in the aqueous extract (PCA) of the pre-coralloid root in spread plate method (**Fig. S6**). No such dispersion was seen in any of the pre-coralloid root extracts of the streak plate method (**Fig. S7**).

Since microscopic observations were carried out in detail to determine the induced hormogonia. As a result, no hormogonia were found induced in any of the extracts of coralloid root (**Fig.S8**), whereas, hormogonia were induced only in the aqueous extract of the pre-coralloid root than that of the other extracts (**Fig.S9**). Even though the dispersion of cyanobacteria towards the disc was clearly seen in the aqueous extract of coralloid and pre-coralloid roots (**Fig. S10 & S11**), no hormogonia development was evidently seen in the aqueous extract of coralloid root at 6th, 8th and 10th days (Fig. 5). Figure 6 clearly showed the hormogonia development in the aqueous extract of the pre-coralloid root at 6th, 8th and 10th days respectively.

Characterization of HIF

Thin-layer chromatography (TLC) analysis of HIF

The acid-hydrolyzed mono and oligosaccharides from the polysaccharide mucilage were subjected to TLC analysis, in which, grey spots were obtained upon spraying sulphuric acid on both the TLC sheets of CA and PCA with Rf values of 0.86 and 0.8 respectively (Fig. 7).

Gas-chromatography and mass spectrometry analysis of HIF

As a result of the GC-MS analysis, the polysaccharide mucilage contribution was only 44.45% in the coralloid root extract (CA) and comparatively greater in the pre-coralloid root extract (PCA) with 71.03%. The monosaccharide units found in the polysaccharide mucilage of CA were arabinose (14.93%), xylose (6.465%), glucose (6.702%), galactose (5.617%), altrose (4. 412%), talose (1.871%), lyxose (1.49%), erythrose (1.305%), mannose (1.087%), ribose (0.874%), rhamnose (0.525%), allose (0.428%), and sorbitol (sugar alcohol) (0.047%) (**Table S3**). Concurrently, the monosaccharide composition of the PCA polysaccharide mucilage was glucose (29.867%), fructose (18.863%), talose (6.737%), lyxose (3.884%), mannose (3.575%), sorbitol (2.469%), allose (2.095%), galactose (1,75%), arabinose (0.855%), ribose (0.726%), sorbose (0.185%), tagatose (0.038%), threose (0.126%) (**Table S4**).

Among the monosaccharide units, fructose, sorbose, tagatose, and threose are absent in CA, similarly, xylose, altrose, erythrose, and rhamnose were absent in the PCA. In CA, the arabinose and galactose content was enhanced than in that of the PCA. In the case of PCA, glucose, fructose, talose, lyxose, mannose, allose, and sorbitol were found in greater quantity than the CA. However, the occurrence of glucose and fructose significantly represents the presence of sucrose in the pre-coralloid root extract (PCE) (**Table S5**) which is absent in the case of coralloid root extract (CA).

Interestingly, the monosaccharide composition of the coralloid root (CA) was 55% of pentose, and 45% of hexose. Unlike CA, the monosaccharide composition of the pre-coralloid root (PCA) was 92% of hexose, and only 8% of pentose (Fig. 8). There was a huge difference in the monosaccharide composition of the coralloid roots during pre- and post-cyanobacterial symbiosis. Therefore, hexoses play a key signaling molecule for the invasion of cyanobacteria in the pre-coralloid root. The same was altered after the cyanobacterial invasion with zero fructose, and high arabinose and galactose content in the coralloid roots to induce N_2 fixation.

DISCUSSION

The coralloid root is a specialized root exclusively for the colonization of diazotrophs, despite, definite knowledge about the origin and transmission of these endophytes still lacking [55]. To date, the most common cyanobacterial symbionts are *Nostoc* or *Anabaena* and *Calothrix*[24,56]. However new genera and species were segregated from the Nostocales in the past decade based on the polyphasic approach [52]. Therefore, studies on the diversity of cyanobiont associated with the coralloid roots of Cycad plants may pave a new path of endo-cyano-symbiosis. In this present study, both the coralloid and pre-coralloid roots were collected from *Cycas circinalis*, a cyanobacterium associated with the algal zone of the coralloid root was isolated and its morphological features were studied.

Upon meticulous microscopic observations, it was found that the cyanobacterium was not *Nostoc* and doesn't meet the criteria of *Nostoc in sensu stricto*. It was observed that the cyanobacterium undergoes a

polymorphic life cycle with different stages such as the *Pseudoanabaena*-like stage, *Nostoc*-like stage, and *Chlorogloeopsis*-stage. Hence, this kind of polymorphic life cycle was already reported in the genus *Cyanocohniella*, which was segregated from the *Nostoc in sensu stricto* in the year 2014 [52]. Very few species were reported in the genus including *Cyanocohniella calida* isolated from the hot thermal spring in the Czech Republic [52], *C. crotaloides* from the Barrier Island, Netherlands at a low temperature of 8°C [53]. *C. rudolphia* isolated from a Potash mining site in Germany [57], *Cyanocohniella* sp. LLY from the Andean Wetlands, South America [58]. Nonetheless, a novel symbiotic cyanobacterium was isolated from the coralloid roots of *Cycas circinalis* in Chennai, India, and hence, named *Cyanocohniella cycadae sp. nov.*

Cyanobacterial endosymbiosis dates back some millions of years ago when cyanobacteria become chloroplast during coevolution in photoautotrophic eukaryotes [59]. Symbiotic mutualism between the host and the cyanobiont results that the former providing shelter to prevent the cyanobiont from predation, and desiccation. Whereas, the latter supplies the host with nutrients [35,36]. In addition, the cyanobiont can synthesize arabinogalactan proteins (AGPs) for the host for its growth and development by cell proliferation, differentiation, and expansion [37,38]. Hence, AGPs play a major role in cell signaling in host-cyanobiont interaction [39,40].

Unlike *Azolla*, (cyanobiont inherited by the next generation) [48], *Cycas* produce strong communication signals for a successful invasion and association with the cyanobiont. Hormogonium-inducing factors are the chemical signaling molecules synthesized by the host plant that induces the cyanobiont to transform it into motile hormogonia [31,42,45]. After successful invasion, the host alters the expression of chemical signaling molecules to induce N_2 fixation in the cyanobiont [45]. Mucilage causes symbiotic interactions by chemo-attracting bacteria to roots [60]. An acidic, slimy, mucilaginous substance in the cortex region of the coralloid roots of *Cycas* is the hormogonium-inducing factor (HIF) [46], similar in the case of *Gunnera* (cyanobiont invasion and association in the nodes) [32]. Sucrose, sucralose, and soluble sugars are the HIF produced by the plant *Gunnera* to induce hormogonia in *Nostoc*[61,62]. Similar kinds of mucilaginous cavities were also reported in the liverworts and hornworts with a low molecular mass of 12 kDa [47]. Simple sugar-based molecules are considered HIF and proven captivating agents in the induction of hormogonia [32,47]. In contrast to this, a crude methanolic extract obtained from the coralloid roots of *Cycas revoluta* had inducted the transformation of *Nostoc* filaments into motile hormogonia. And the resultant HIF was identified as diacylglycerol 1-palmitoyl-2-linoleoyl-sn-glycerol [63]. Moreover, Lobakova et al. [64] reported the presence of phenolic compounds in the cyanobacterial zone of the coralloid roots, and functions as a rich source of antimicrobial compounds to mediate stability with the associated symbiosis [27]. Still, there is no clear idea of the signaling molecules and their interactions between the host and cyanobiont [65,66].

In the present case, among the aqueous, ethanol, and hexane extracts of both the coralloid and pre-coralloid roots, the aqueous extracts of coralloid and pre-coralloid roots were shown to attract

cyanobionts towards the disc. Concurrently, hormogonia induction was only seen in the pre-coralloid root extracts (PCA). Upon acid hydrolysis and GC-MS analysis, the mucilage constitutes 44.5% monosaccharide composition in the coralloid extract, whereas, the same was 71.03% in the pre-coralloid root. Arabinose (14.93%), galactose (5.61%), xylose (6.46%), glucose (6.70%), and altrose (4.41%) were the major monosaccharides derived from the coralloid roots. Glucose (29.86%), fructose (18.86%), talose (6.73%), and lyxose (3.88%), were the most common monosaccharide units in the pre-coralloid root. In comparison, fructose was absent in the coralloid root, and galactose was found very least in the pre-coralloid root. Simultaneously, the hexose and pentose content in the coralloid root was 45% and 55% respectively. The same was 92% and 8% in the case of the pre-coralloid root. As a result, it has been considered that the hexoses are the signaling molecules for the induction of hormogonia in the pre-coralloid roots. Reduction of hexose and increase in pentose (arabinose and galactose) suppresses hormogonia induction and enhances N_2 fixation.

In support of this present study, Bürgmann reported that the sugar molecules are the only candidates that induce nitrogen fixation, and artificial supplements of single sugars induced nitrogen fixation in diazotrophs [67]. Glucose, galactose, and arabinose were utilized by the diazotrophs for their growth and nitrogen fixation [68]. Galactose and arabinose were the most common constituent of Rice plant exudates resulting in induced N2 fixation in diazotrophs and enhanced plant growth [69]. Carbohydrate-rich mucilage secretion was found in the aerial roots of Sierra Mixe maize grown in nitrate-depleted soil and was rich in diazotroph diversity resulting in higher atmospheric nitrogen fixation (82%). The monosaccharide composition of the mucilage from Sierra Mixe maize constitutes fucose (41%), galactose (36%), and arabinose (14%) [70–72]. Fucose, xylose, arabinose, and galactose were the most common monosaccharides in the mucilage of Sierra Mixe aerial roots [73]. According to the present study, the coralloid root is rich in arabinose, galactose, xylose, glucose, and altrose. The root mucilage was reported to maintain low oxygen levels in a micro-aerobic environment to induce nitrogenase activity[72]. Therefore, it is evidently clear in this study that the pre-coralloid root is rich in hexose to induce hormogonia and invasion, and pentose in the coralloid root enhances heterocyst formation (N_2 fixation) in the cyanobiont.

About 50–70 teragrams of nitrogen are fixed annually by associative N-fixing bacteria worldwide (Tg). Symbiotic diazotrophs in plant organ cells like root nodules utilise the host's photosynthetic outputs as energy sources and fix N to aid in the growth and development of the host [74,75]. In future, a viable strategy for supplying biologically fixed nitrogen in order to boost agricultural productivity is to engineer an artificial N-fixing symbiosis between diazotrophic bacteria and host crop plants [76–78].

CONCLUSION

In this context, the mucilage secretions in the coralloid (CA) and pre-coralloid roots (PCA) were extracted and screened for the induction of hormogonia in a novel cyanobacterium *Cyanocohniella cycadae sp. nov*. The polysaccharide extracts of both the CA and PCA were acid hydrolyzed, derivatised and characterized by GC-MS analysis. As a result, CA mucilage polysaccharide was majorly composed of

arabinose, galactose, xylose, glucose, and altrose, whereas the PCA mucilage polysaccharide constitutes of greater quantity of glucose, fructose, talose, and lyxose. Intriguingly, hexose was found greater in PCA mucilage polysaccharide, and pentose was higher in the case of CA. Therefore, as a concluding result, the mucilage found in the pre-coralloid root induces the hormogonia development in cyanobiont to invade the coralloid root. At the same time, the mucilage in the coralloid root suppresses hormogonia induction and supports nitrogen fixation by enhancing heterocyst development in the cyanobiont. Hence, this investigation sheds light on the alteration of mucilage signaling molecules in the plant host and the regulation of induction/suppression of hormogonia in the endosymbiotic cyanobiont. Henceforth, more studies are needed to elucidate the biosynthetic pathways of mucilage secretion in the host and regulation on the morphology of cyanobacterium and its life-cycle.

Declarations

Author contribution

This present study was assigned as a Post graduate research work to Ms. Kreedika and Mr. Anton Rejoy by Prof. S. Elumalai under the guidance of Dr. Rajesh Kanna, Dr. Rajesh, and Dr. Preethy. The manuscript was drafted by Dr. Rajesh Kanna, corrected by the corresponding author and approved by all the co-authors.

We confirm that all methods were performed in accordance with the relevant guidelines/regulations/legislation.

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We are grateful that the administrators of the Horticulture Farm at the Madhavaram Botanical Garden in Madhavaram, Chennai, India, gave us permission to collect the coralloid and pre-coralloid roots from the Cycas plant.

DATA AVAILABILITY

The data can be obtained upon request from the corresponding author, Prof. S. Elumalai.

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Figures

Figure 1

A) The gymnosperm plant *Cycas circinalis* grown in the Horticulture Farm in Madhavaram Botanical Garden, Madhavaram, Chennai, Tamil Nadu, India; B) Roots that are coralloid and pre-coralloid are collected; C & D) Coralloid and Pre-coralloid (absence of cyanobacterial colonisation) roots derived from the Cycas plant respectively.

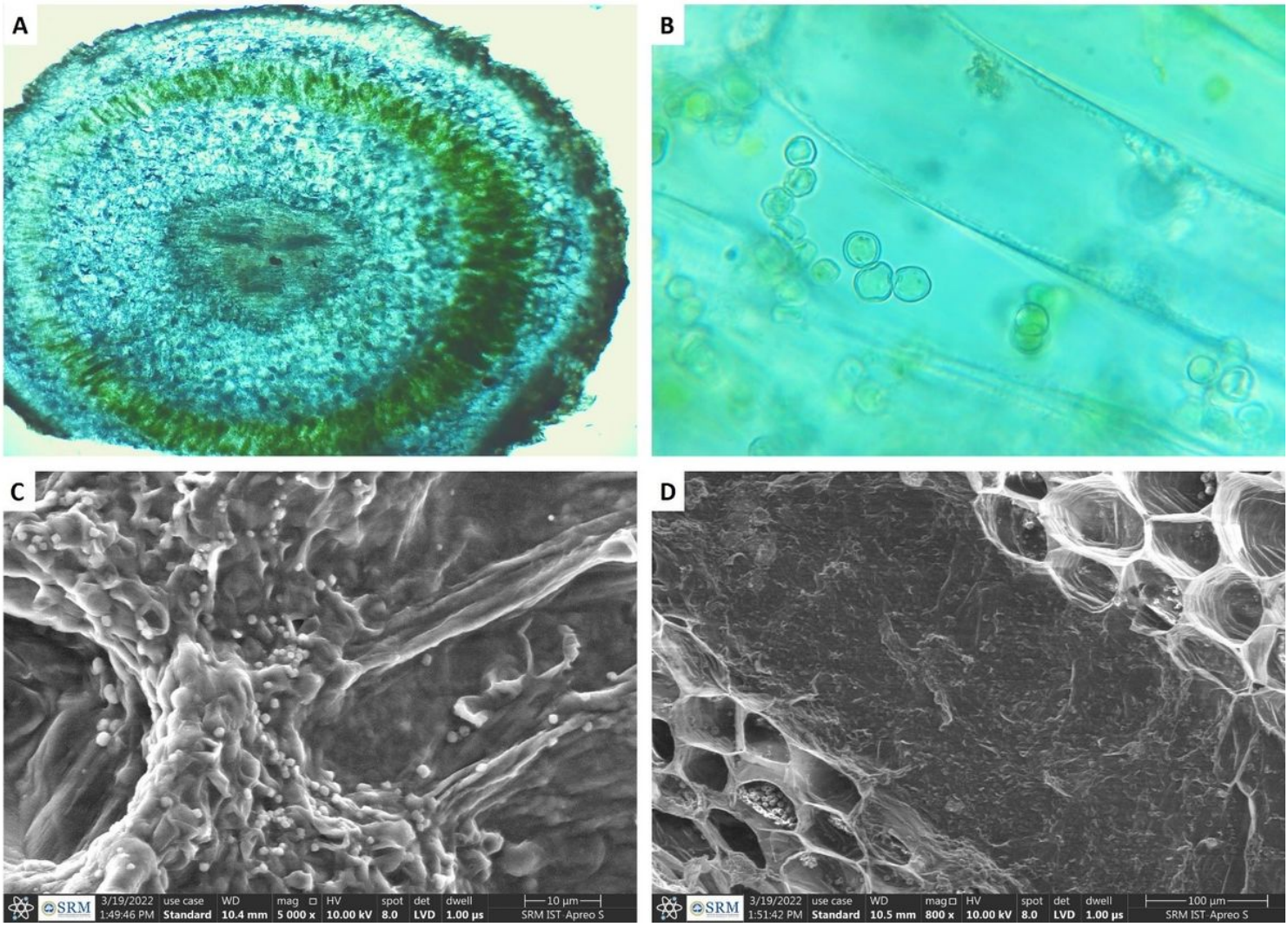

Figure 2

A) A radial arrangement of aerenchyma cultivating symbiotic cyanobacterium may be seen in this thin cross-section of the coralloid root of *Cycas circinalis* at a 4X magnification; B) Inside the aerenchyma, cyanobacterium is growing (40 X magnification); C & D) Cross-sectional views of the coralloid roots of the Cycas plant taken using a scanning electron microscope (SEM) reveal vacant aerenchyma that has been colonised by the symbiotic cyanobacterium.

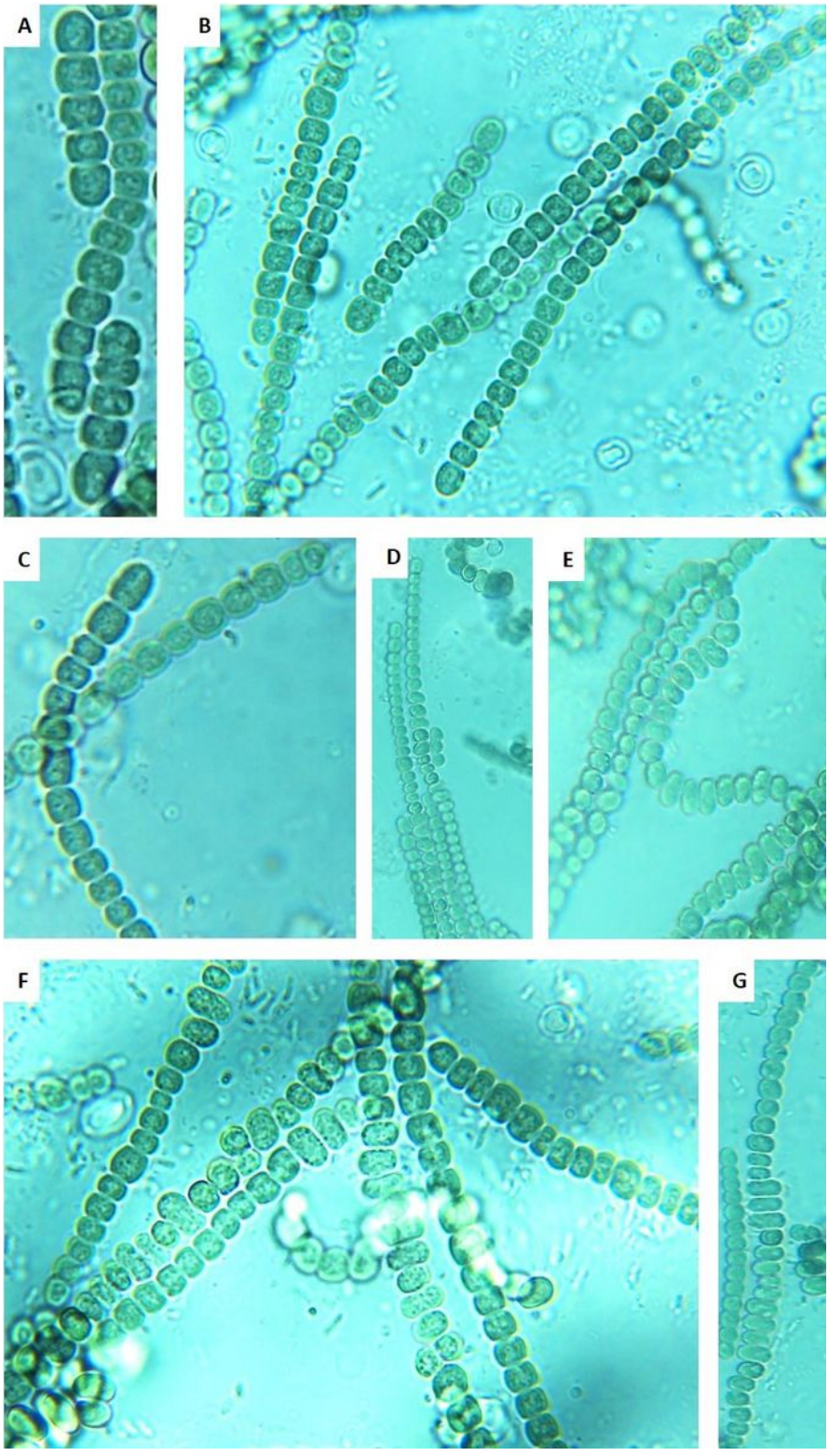

Figure 3

The symbiotic cyanobacterium was found at several life cycle phases in the coralloid root of *Cycas circinalis*; A & B) Hormogonia stage; C) *Pseudoanabaena*-like stage; D-G) *Nostoc*-like stage.

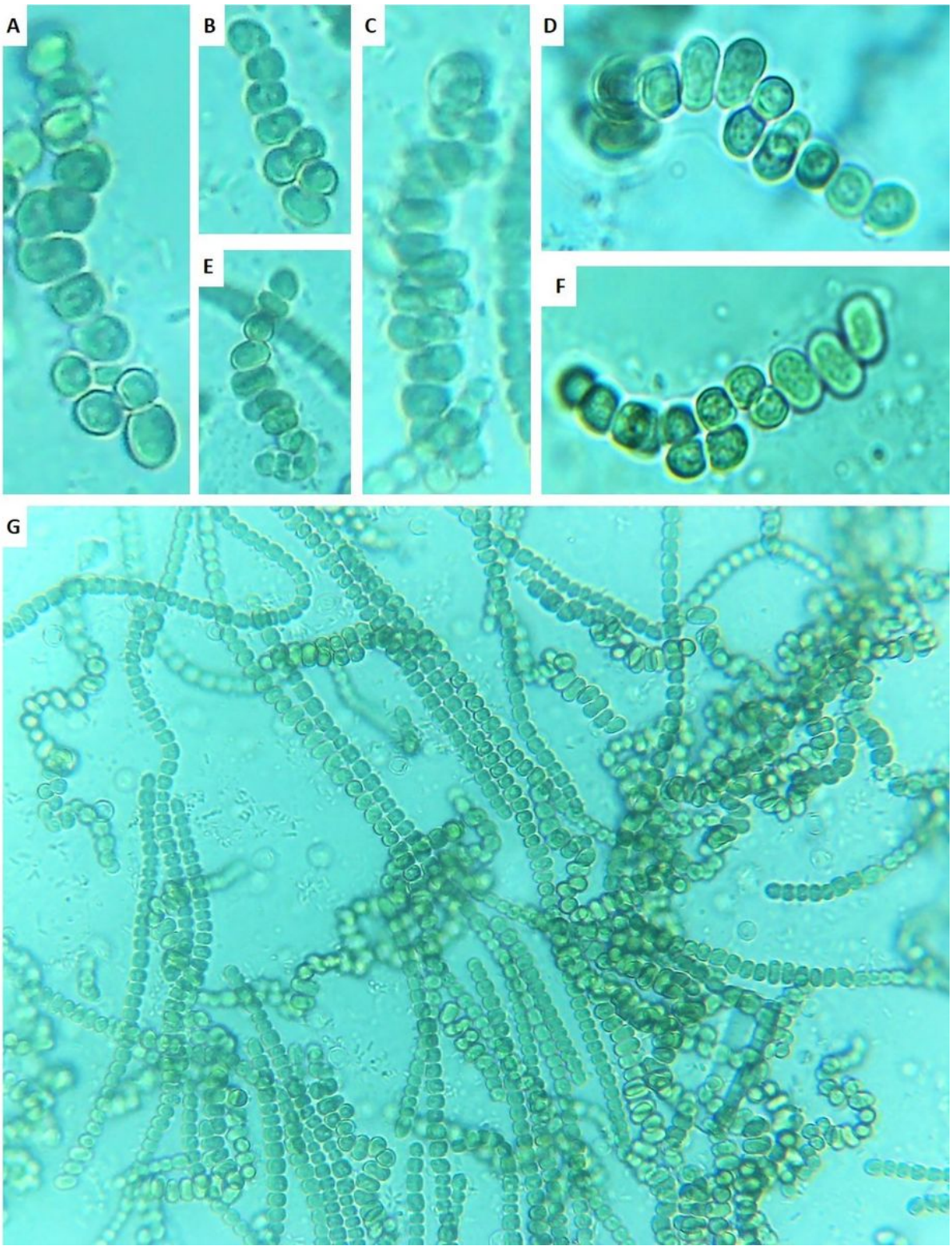

Figure 4

A-F) *Chlorogloeopsis*-like stages; G) The symbiotic cyanobacterium isolated from the coralloid root of the *Cycas circinalis* has gone through every stage of its life cycle.

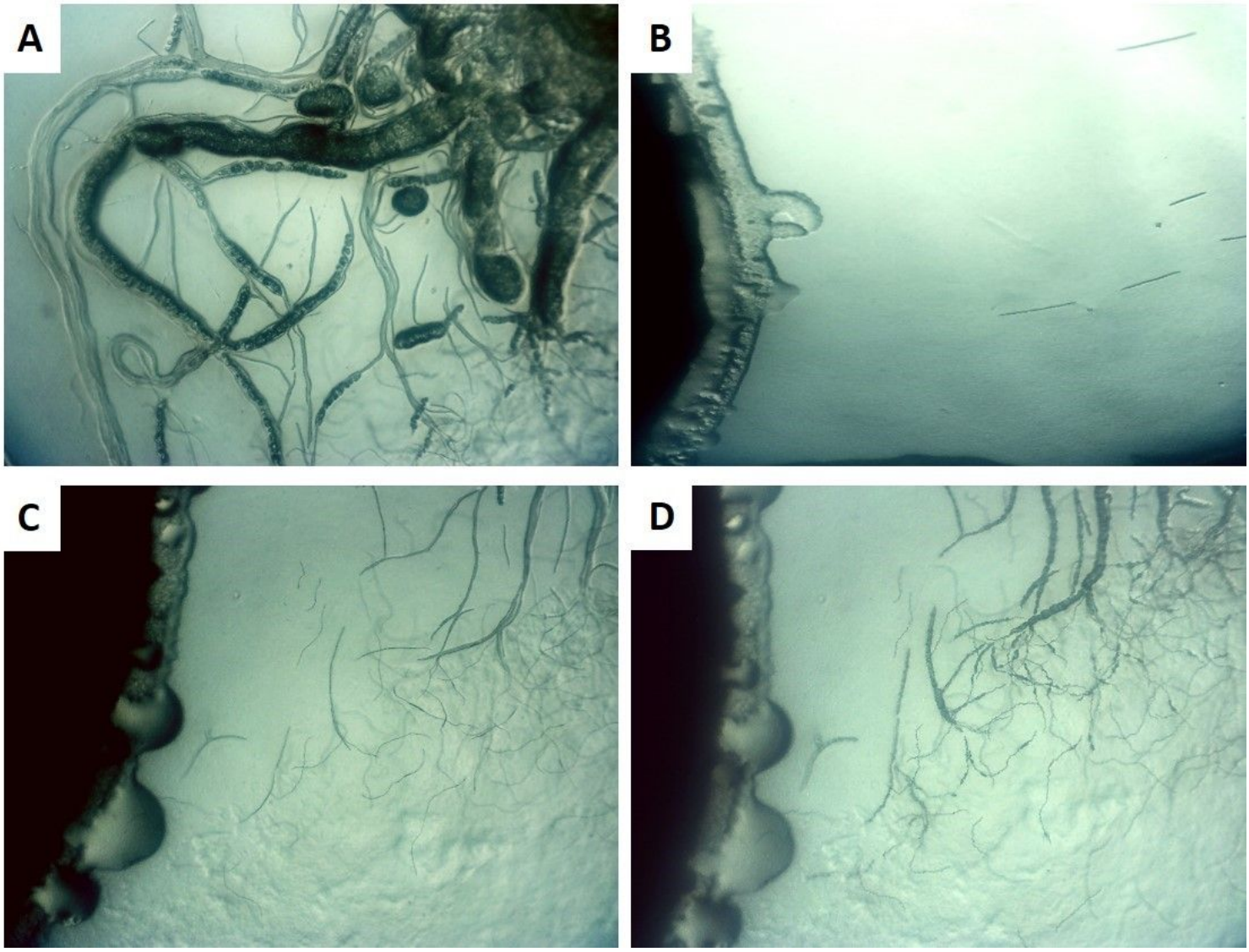

Figure 5

Screening for hormogonium-inducing factor in the coralloid root of Cycas plant; A) Control showing no hormogonium; B) After six days of incubation, the filaments are moving in the direction of the disc.; C & D) Despite no hormogonium being generated, cyanobacterial filaments were moving toward the disc on the eighth and tenth day of incubation.

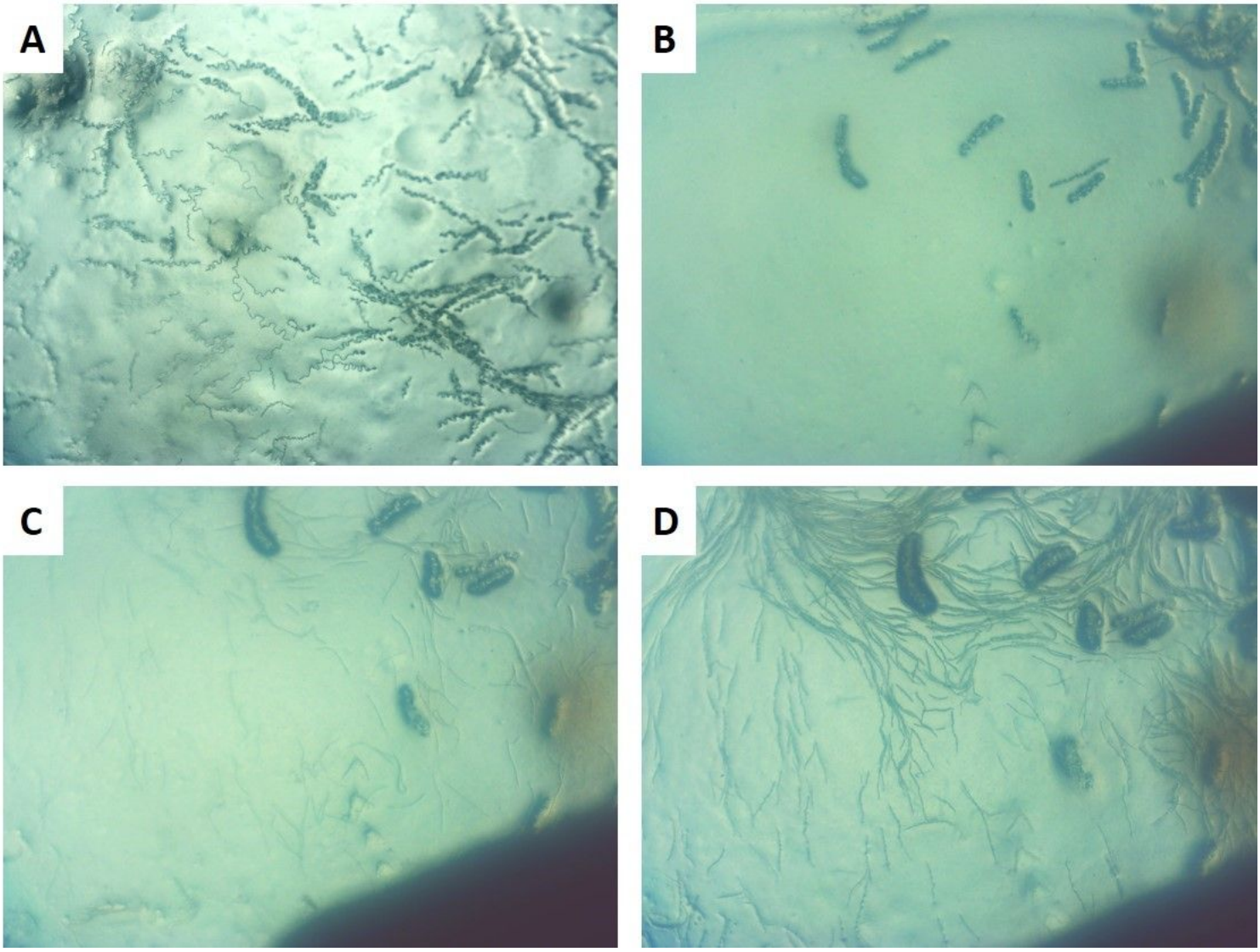

Figure 6

Screening for hormogonium-inducing factor in the pre-coralloid root of Cycas plant; A) Control showing no hormogonium; B, C & D) *Cyanocohniella cycadea*'s induced hormogonia toward the disc is seen on days six, eight, and ten of incubation.

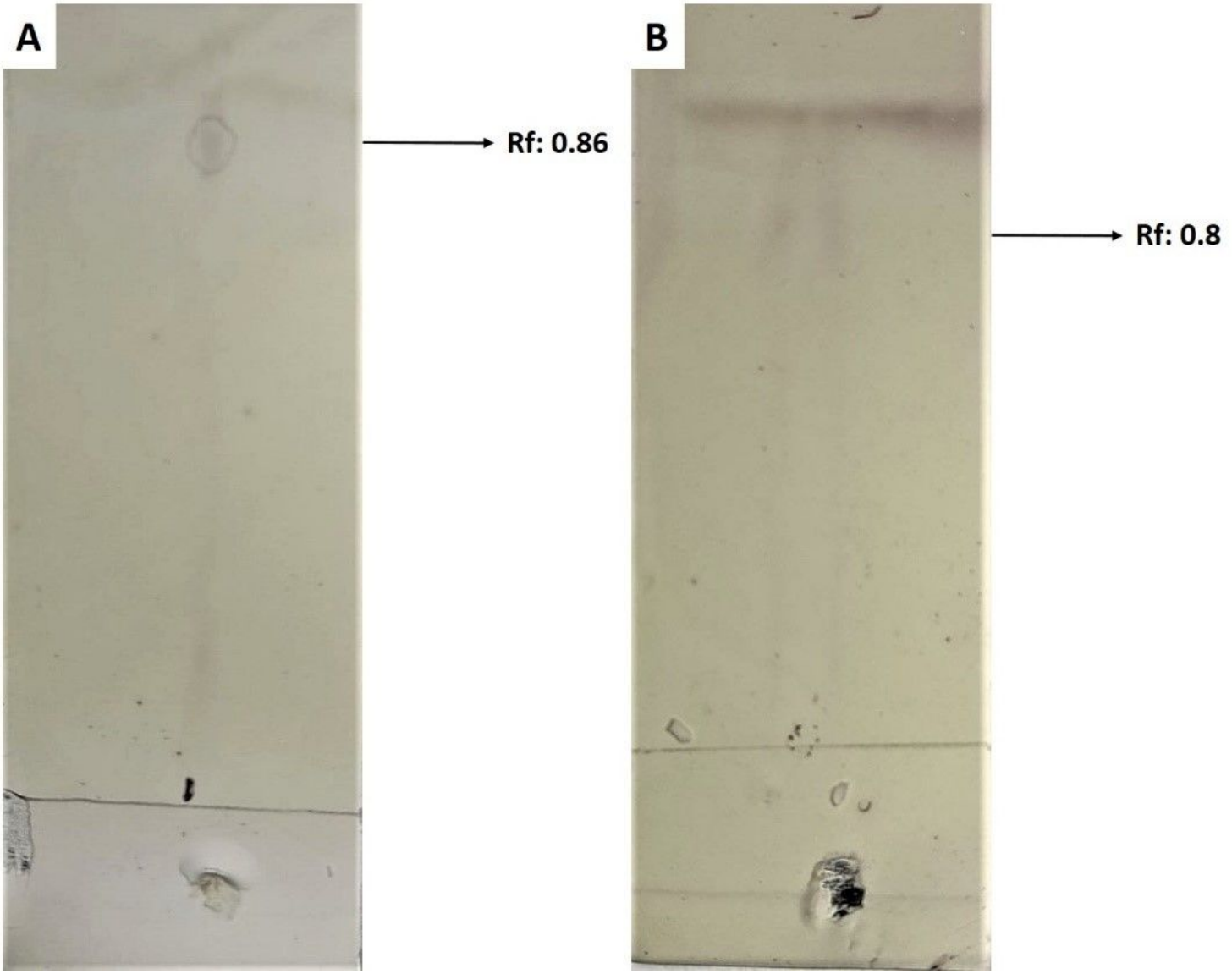

Figure 7

TLC sheets displaying spots of acid-hydrolyzed mucilage from the aqueous extract of both A) coralloid and B) pre-coralloid roots, respectively.

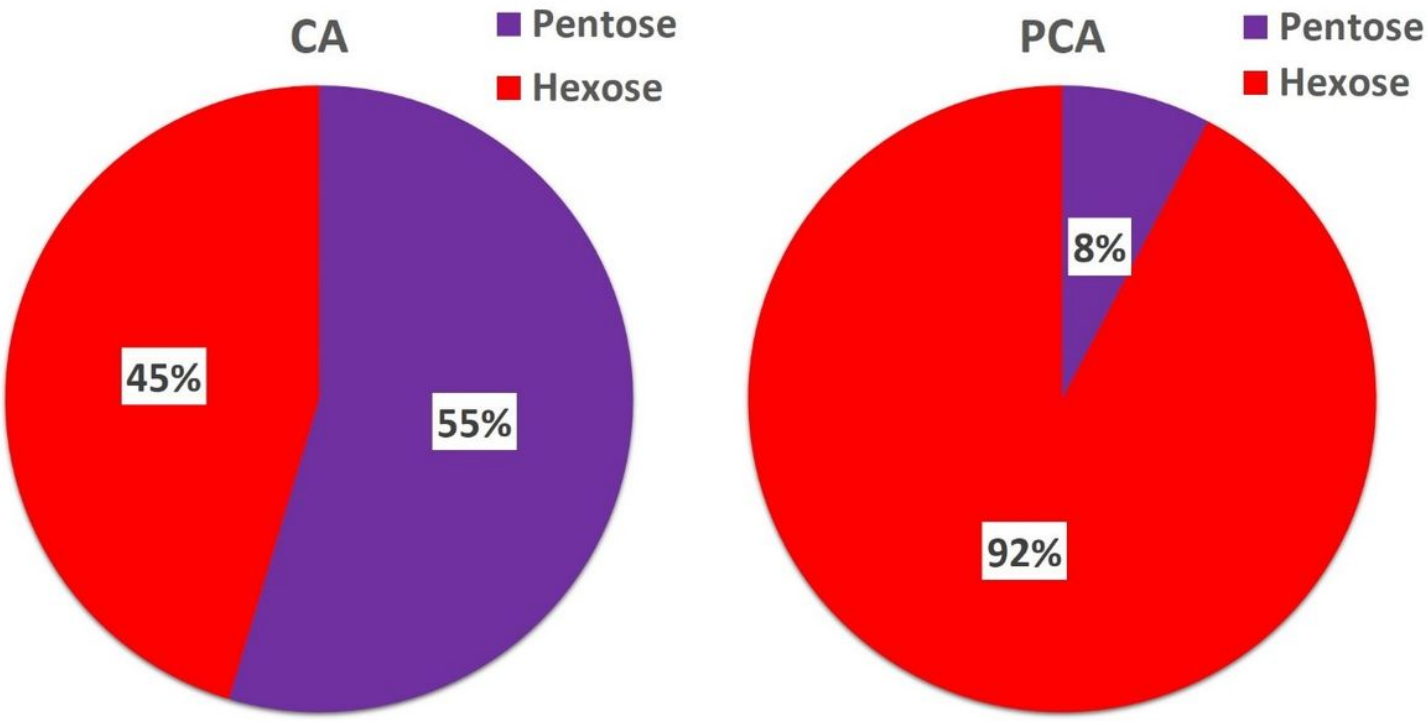

Figure 8

The two pie graphs illustrating the distribution of pentose and hexose in the mucilage of both the aqueous extract of PCA (coralloid root) and CA (coralloid root) (pre-coralloid root).

Supplementary Files

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