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Basidiomycete *(Pseudolagarobasidium acaciicola)* in coconut *(Cocos nucifera)* suspension culture — a report

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Coconut (*Cocos nucifera* L.), a monospecific woody tree is recalcitrant to *in-vitro* culture. Doubling the yield through technological interventionshas been reported in many palm species (Manorama *et al.,* 2019). Even though most of the *in-vitro* protocols use solid media, suspension cultures utilizing liquid media for establishing embryogenic cells with high regenerative capacity is reported (Al-Khayri, 2012). As the liquid media provide uniform conditions of nutrient availability to explants, mass multiplication of coconut embryogenic cells from plumular explant via suspension culture was attempted.

The explants used were plumules from 10-month-old nuts, harvested from 50-year-old palms of West Coast Tall variety planted at ICAR - CPCRI, Kasaragod, Kerala. The endosperm plugs were scooped out from the dehusked coconuts and sterilized with mercuric chloride, 0.1% (w/v), for three minutes. The embryos were excised and further sterilized using 20% sodium hypochlorite solution for 15 minutes and washed using double-distilled autoclaved water 5-6 times. Plumules are extracted from embryo and were inoculated in Y3 medium with 16.5 mg/l 2,4-Dichlorophenoxyacetic acid, 1 mg/l Thidiazuron, 0.1% (w/v) activated charcoal. The gelling agent used was agarose @ 7.5g/l. The plates were kept in dark condition at 27°C. After three weeks of inoculation, callus initiation was observed and small quantity of friable calli, whenever obtained was used for initiating suspension culture. Suspension culture medium was Y3 medium supplemented with biotin 1 mL/L, myoinositol 100 mg/L, malt extract 100 mg/L, L-glutamine 100 mg/L, sucrose 30 g/L supplemented with auxin picloram @ 1mg/L and activated charcoal @ 1 g/l. The suspensions were placed in an orbital shaker (Lab-line orbit environ - shaker, India) @ 100 rpm, 27°C temperature in dark condition. The friable callus tissues inoculated into suspension culture media dissociated into small granular clumps which were then plated on suspension medium gelled using agarose @ 7.5 g/L (Figs. 1-6).

Semi-transparent outgrowth was observed in few clumps which resembled friable calli of coconut plumular explants. Microscopic observations (Diaplan Microscope, LeitzWetzlar Type 307-148.002 at magnification of 40x, stained with acetocarmine) showed that these cultures contained two types of cells: longer (about 20 µm) and shorter (about 2 µm). The big cells were angular and elongated while the small were mostly circular in shape. The internal cellular components were visible in larger cells and in certain slides smaller cells appeared to ooze out from these larger cells (Fig. 6).

For identification of fungus, it was grown in 1.5 ml eppendorf tube containing 0.5 ml of Sabouraud Dextrose Broth supplemented with chloramphenicol and incubated in an orbital shaker (Innova, USA) at 150 rpm and 30°C for 4-5 days. The mycelia were then harvested by filtration and mechanically disrupted followed by genomic DNA extraction using Invitrogen gDNA isolation kit. Manufacturer's instructions were followed which was based on selective binding of dsDNA to silica-based membrane and subsequent elution of DNA in low salt Elution Buffer. The quality and quantity of gDNA was checked by Nanodrop ND8000.

Extracted DNA was amplified using an Eppendorf Master Cyclerthermal cycler (Eppendorf, US). The primers used were ITS1 (5′ TCC GTA GGT GAA CCT GCG G 3′) and ITS4 (5′TCC TCC GCT TAT TGA TAT GC 3′) which hybridize at the end of 18S rDNA and at the beginning of 28S rDNA, respectively. The PCR mixture (50 µl) contained 10 µl of DNA template, 6 µl of 25 mM $MgCl_2$, 5 µl of PCR buffer without $MgCl_2$; 200 µM each deoxynucleoside triphosphate, 25 pmol each of forward and reverse primer and 1 U of Taq

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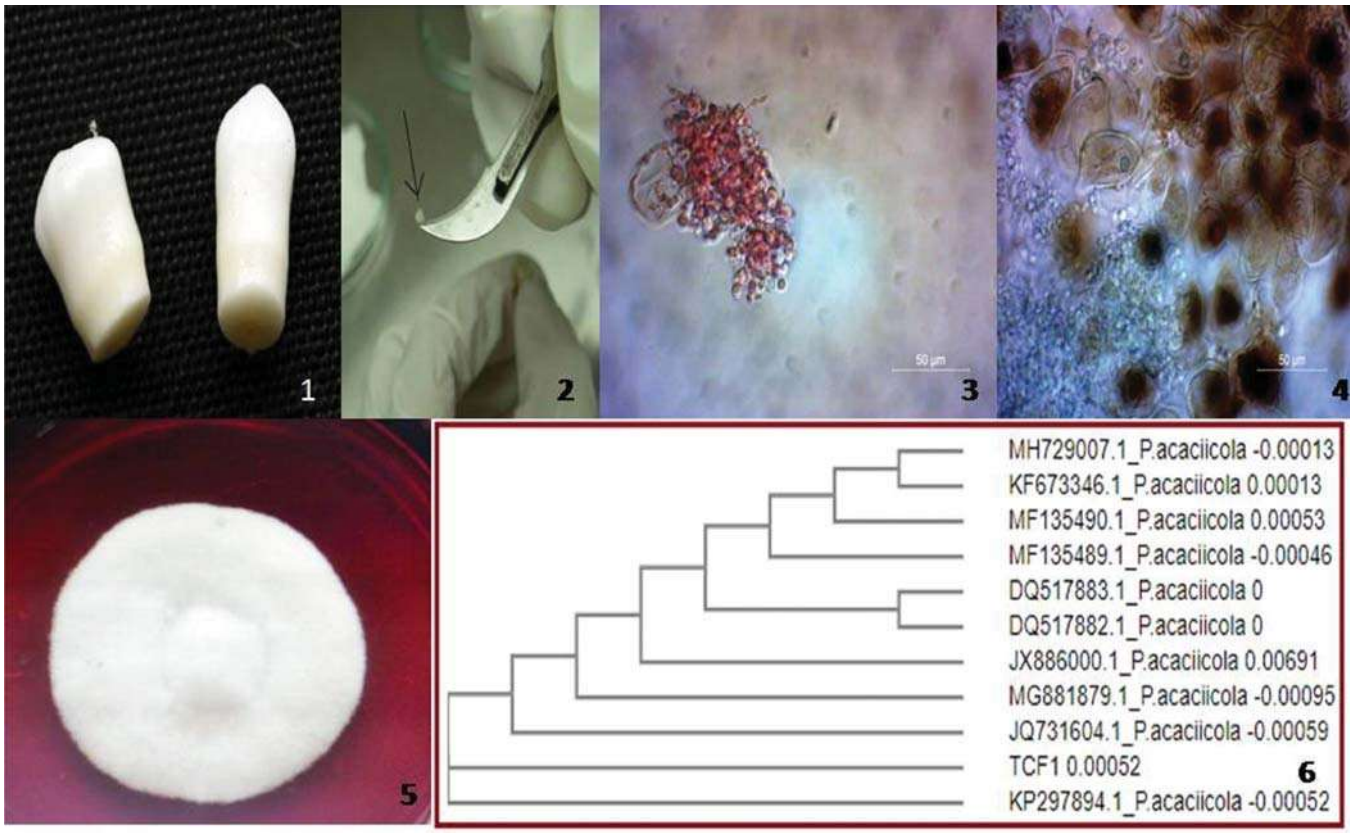

Fig. 1: Coconut embryos; **2.** extracted plumule; **3.** microscopic observation; **4.** two different types of cells; **5.** fungus isolated and purifed on Martin's Rose Bengal Agar medium; **6.** phylogeny of *Pseudolagarobasidium acaciicola* TCF1.

DNA polymerase. Reactions involved 1 cycle at 95°C for 5 min, followed by 35 cycles with a denaturation step at 95°C for 30 s, an annealing step at 55°C for 1 min, and an extension step at 72°C for 1 min, followed by 1 cycle at 72°C for 6 min.

A negative control of sterile water was included. The amplicon was electrophoretically separated in 1% agarose gel in 1× Tris-borate-EDTA buffer and visualized using ethidium bromide under UV illumination and its concentration was checked in a Nanodrop ND 8000. Molecular weight ladder (100-bp DNA) was included in the run. The amplified DNA from PCR was purified using PureLink PCR Purification Kit (Invitrogen) as specified by the manufacturer.

The amplicon was cycle sequenced with forward and reverse primers using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) in ABI 3730×l DNA Analyzer. After sequencing, forward and reverse rDNA sequences were assembled and contig was generated after trimming the low quality bases. The generated contig was analyzed by using the BLAST alignment program of the GenBank database (National Institutes of Health). The computer alignment provided a list of matching organisms, ranked in order of similarity between unknown sequence and sequence of corresponding organism from database.

The rDNA sequence analysis using Basic Local Alignment Search Tool (BLAST) of National Centre for Biotechnology Information (NCBI) demonstrated that it was derived from the fungal ITS regions and revealed the organism to be *Pseudolagarobasidium acaciicola*. The sequence was deposited in NCBI Genbank database with Accession number MK163558. Based on maximum identity score, first few sequences were selected and aligned using Clustal Ω and a dendrogram was constructed.

Based on homology and phylogenetic analysis, fungal hyphae growing in *in-vitro* cultured coconut plumular cell clumps belonged to a monophyletic genus, *Pseudolagarobasidium,* in *Polyporales* clade. The fungus *Pseudolagarobasidium acaciicola,* reported here, was first described from South Africa as a plant pathogen. It was widely used as a myco herbicide to control the invasion of Acacia species in South Africa (Barathikannan *et al.,* 2017).

The contaminations occurring during coconut meristem culture was reported (Neema *et al.,* 2022). But this is the first report of basidiomycete, known for white rot, thriving inside living coconut tissues maintained on artificial medium. Another noticeable

fact was that unlike the fungal contamination in suspension cultures, which forms ball-like structures when suspensions are incubated in the shakers, the clumps observed in the study was grain like, much like the suspension cell clumps, which might mislead the researchers.

This resupinate fungus,though has an ecological range spanning from saprotrophy to parasitism, is also reported as an endophyte of healthy, living cocoa trees (Crozier *et al.,* 2006). Largely known as a saprophyte, its existence in coconut host could be seen as a survival strategy switching from one mode to another, indicating its nutritional plasticity and hence, ecologically important. In conclusion, this is to our knowledge the first reported case of the presence of *Pseudolagaro-basidium acaciicola* in coconut *in-vitro* suspension culture.

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