MethodsX 16 (2026) 103969

Contents lists available at ScienceDirect

MethodsX

journal homepage: www.elsevier.com/locate/methodsx

A rapid and simplified technique for inducing *Ganoderma lucidum* infections in coconut and arecanut seedlings

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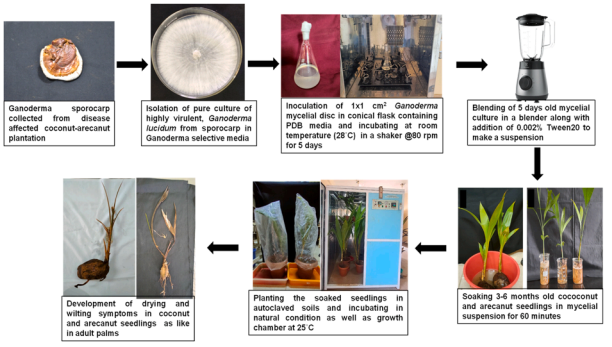
GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Coconut
Arecanut
Ganoderma
Mycelia
Inoculation

ABSTRACT

Ganoderma lucidum is a soil borne fungi known to cause Basal stem rot (BSR) or Thanjavur wilt or *Ganoderma* wilt disease in coconut as well as Foot rot or *Anabe roga* disease in arecanut plantations. Due to its soil borne nature, effectively managing the disease in field conditions is highly challenging. To combat this issue, the development of resistant or tolerant seedlings is crucial for sustainable production of coconut and arecanut palms. Therefore, a reliable and rapid method to assess resistance of palms to *Ganoderma* disease is essential. Here we report a rapid inoculation technique wherein seedlings were soaked in 5 days old mycelia cultures supplemented with 0.002% Tween 20 for a period of 60 min. The initial symptoms could be noticed 15 days after

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https://doi.org/10.1016/j.mex.2026.103969
Received 2 December 2024; Accepted 21 May 2026
Available online 22 May 2026

inoculation in arecanut and 30 days after inoculation in coconut. The technique ensures consistent infection, allowing disease evaluation as early as two weeks post inoculation with *G. lucidum*. Our results indicate that this new inoculation technique can be used routinely to screen coconut and arecanut seedlings for BSR disease and to evaluate the resistance of different cultivars to *G. lucidum*.

- Developed a rapid, simple and efficient inoculation technique.
- The technique was evaluated for coconut and arecanut seedlings as well as tissue culture plantlets.

SPECIFICATIONS TABLE

Subject area	Agricultural and biological sciences
More specific subject area	Screening technique, plant microbe interaction
Name of your method	Mycelial inoculation technique
Name and reference of original method	Maria IP, Delia A, Cahya P & Antonius S. 2018. A Rapid Inoculation Method for Infection of Ganoderma in Oil Palm. Int.J. of Oilpalm.1(1):1–9.
Resource availability	Reagents and Equipment are listed in the Materials section

Background

Coconut (*Cocos nucifera*) and arecanut (*Areca catechu*) are perennial plantation crops cultivated in several tropical countries. However various diseases hinder in crop production and limit palms productivity per unit area. In coconut, bud rot, ganoderma wilt and root (wilt) are the most prominent diseases causing economic loss [1]. Similarily, in arecanut cultivation, mahali, yellow leaf disease and ganoderma wilt are the major diseases responsible for limiting production [2]. Ganoderma Wilt, in particular, has emerged as a major threat to the sustainability and productivity of both coconut and arecanut plantations in India. Initially reported in the southern states, the disease has recently been observed spreading to northeastern states, especially Assam and Meghalaya [3]. Ganoderma wilt disease causes a decrease in fresh fruit bunch (FFB) weight and eventual death of the palm.

The *Ganoderma* pathogen is challenging to control as it resides and spreads in the soil, taking a long time to exhibit visible symptoms on palms. Although various management strategies, including mechanical, chemical, and biological methods, are available, the disease remains difficult to control for this reason. Breeding offers a potential alternative for disease management by developing resistant or tolerant genotypes. However, a proper, simple, and rapid screening methodology for assessing *Ganoderma* pathogenicity is still lacking.

Currently, the most commonly used artificial inoculation method for *G. lucidum* in coconut involves colonized sorghum grains [4]. This method has been widely adopted as the standard technique for inoculating palms in BSR disease research. However, it is both labor-intensive and time-consuming. A typical sorghum grain inoculation requires at least six months from preparation to disease evaluation. Moreover, due to the long incubation period and challenges in sterilizing grains, the contamination rate by other fungi is relatively high.

In coconut, the sorghum seed inoculation technique takes approximately 6 to 18 months for visible symptom development. A similar situation exists for arecanut, although artificial inoculation techniques in arecanut have not been extensively studied. The prolonged symptom development period makes disease resistance screening a significant challenge. Additionally, the use of 1.5- to 2-year-old seedlings in the sorghum seed method adds to the difficulty, as seedlings must be nurtured for an extended period before inoculation, only to succumb to the pathogen afterward. This not only lengthens the process but also increases labor requirements and necessitates substantial experimental space over a prolonged duration.

Given these challenges, our team has developed a simple and rapid inoculation methodology. This new approach aims to streamline and accelerate routine screening for BSR disease in coconut and arecanut seedlings, effectively addressing the time and space limitations associated with traditional methods.

Method details

Materials

- 60 mm x 15 mm sterile plastic Petri dishes.
- Forceps, FisherbrandTM filter/membrane forceps or similar (Fisher Scientific #09–73–50).
- 250 ml glass beakers (Kimax, USA, No. 14,000).
- 1000 µl pipette tips with filters (USA Scientific, 1122–1830), sterile.
- Potato Dextrose Agar media (Granulated, GMH096, HiMedia or similar).

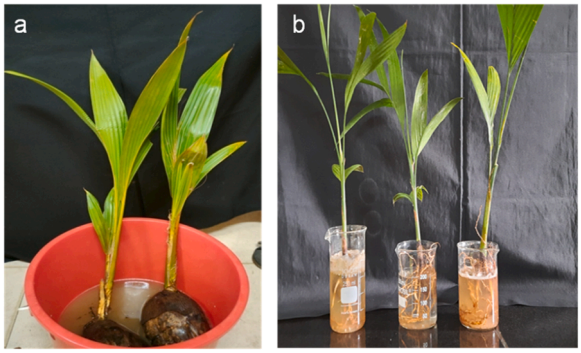

Fig. 1. Mycelial suspension inoculation technique- soaking of 6 months old coconut (a) and arecanut (b) in Ganoderma mycelial suspension supplimented with 0.002% Tween® 20 for 1 hour.

- Conical flask 2 L for propagating fungal inoculants (Borosil, HiMedia or similar).
- 1 ml pipette tips (USA Scientific, 1051–0360), sterile.
- Tween20 (SRL, CAS: 9005-64-5).
- Sterile hand held blender (Panasonic® HR1603 or similar).
- 20 L beaker or plastic bucket for soaking the seedlings.
- Temperature controlled shaker (BR Biochem Orbital Incubator Shaker, Speed 20 RPM to 300 RPM, BRI-111).
- Potting mixture (2:1:1 soil, sand and FYM).
- Plastic planting pots (11 × 10 × 6.3 inch; L x W x H; from Ahalya Nurseries, India)[Six 5 mm holes drilled in the bottom of each pot to allow water uptake and drainage.
- Growth chamber with temperature and humidity control facility(large enough to keep seedlings inside -customized).
- Coconut and arecanut seedlings (3-4 months old).

Procedure

G. lucidum isolate of G9 (NCBI accession number: OQ381241) used in this study were isolated from diseased palms of coconut from Kasaragod districts of Kerala during 2018. Ganoderma selective media (GSM) was used for isolation of Ganoderma from infected root and soil. The pure culture was then maintained on potato dextrose agar (PDA) at 25°C for further studies. Coconut seedlings (*West Coast Tall*, 3–6 months old) and arecanut seedlings (*South Kannara Local*, 3–6 months old) were used for the experiment. A total of 25 coconut seedlings and 50 arecanut seedlings were used in replicated trials. Additionally, 10 tissue culture plantlets of both coconut and arecanut were included for screening.

Preparation of GSM used for isolation of Ganoderma

Material required

Part A: Bacto peptone-5 g, K_2HPO_4–0.5 g, Agar 20 g, Distilled water-900ml.

Part B: Streptomycin sulphate-300 mg, Chloramphencol-100 mg, PCNB-285 mg, Ridomil (25% WP)-130MG, Benlate T20–150 mg, Ethanol 95%−20 ml, Lactic acid50%−2 ml, Tannic acid-1.25 g, Distilled water-80 ml.

Procedure

- Sterilize Part A ingredients at 121°C for 20 min using Autoclave and store it upon cooling.
- Dissolve the part B ingredients in distilled water by continuous shaking for 10–15 min.
- Heat Part A until the agar is completely dissolved. Allow it to cool slightly until it reaches a bearable temperature, then mix in 20 ml of Part B into part A and pour the resultant media into the desired petri plates for isolation.

Inoculation of G. lucidum to seedlings and tissue culture plantlets

Artificial inoculation of *G. lucidum* was performed using mycelium inoculation technique [5] with some modifications. The inoculum of *G. lucidum* isolate G9 was prepared by cutting mycelia from a 6-day-old PDA culture into 1 × 1 cm^2 plugs using a sterile scalpel. These plugs were then inoculated into 100 mL of Potato Dextrose Broth (PDB) and incubated at 28°C with continuous agitation at 80 rpm for five days. After incubation for 5 days, the mycelium cultures were blended using a sterile hand held blender (Panasonic® HR1603) for 3 min with the addition of 0.002% Tween® 20. The concentration of mycelia was adjusted to 10^5 fragments mL^{-1} using a hemocytometer. For inoculation, 3–6-month-old seedlings of the *West Coast Tall* (WCT) coconut variety and the *South Kannara Local*

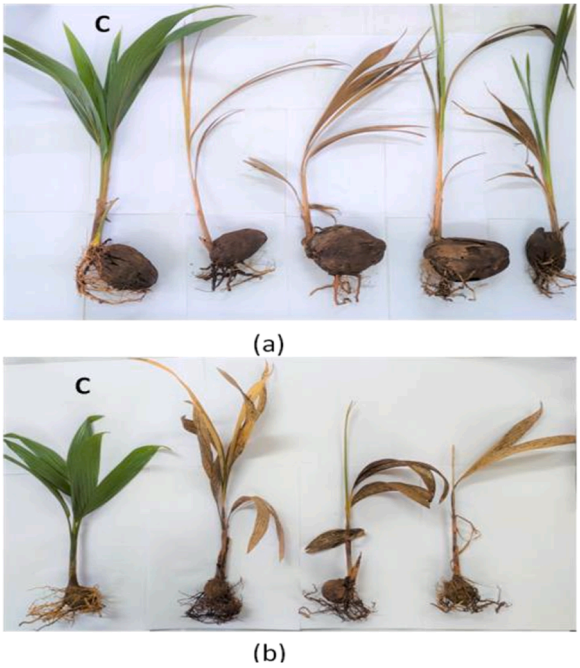

Fig. 2. Symptoms of basal stem rot disease in coconut (a) and arecanut (b) seedlings to *Ganoderma lucidum* after soaking in mycelial suspension solution supplemented with 0.002% Tween® 20 for 1 h. Seedlings soaked in PDA suspension supplemented with 0.002% Tween® 20 alone served as control.

Fig. 3. Different symptoms observed in coconut and arecanut seedlings after inoculation with *G.lucidum* mycelia suspension (a)& (b) Excessive rotting of the stem and root portions, (c) &(d) Browning and rotting in longitudinal section of stem and root, (e) rotting and appearance of mycelia growth on basal stem portion.

(SK-Local) arecanut variety were carefully uprooted from the soil and thoroughly washed. The roots were then immersed in the prepared mycelium suspension for 60 min before being replanted (Fig. 1). This process ensured thorough exposure of the seedling roots to the mycelium suspension, facilitating successful infection. Control seedlings were treated similarly but immersed in sterile water or PDB for 60 min instead. The inoculated coconut and arecanut seedlings were then replanted in sterile soil-filled pots and grown in a glasshouse. The soil was sterilized using an autoclave (liquid cycle: 121°C, 20 min, 100 kPa), followed by cooling to room temperature for two days. After this resting period, the soil was sterilized again under the same conditions before potting. The potted seedlings were maintained in a greenhouse with an ambient relative humidity of 60–80% at 30°C and watered twice daily. Another set of seedlings was placed in a growth chamber at 28°C with 70–80% relative humidity. Disease symptom progression was monitored weekly, with each seedling planted in a separate pot.

A concentration of 10^5 mycelial fragments mL^{-1} of *G. lucidum* effectively infected the plant roots and ensured consistent pathogenicity. Due to the weak soil competitiveness of *G. lucidum*, precise inoculum quantification was crucial for achieving successful infection.

The same methodology was applied to study the pathogenicity of *G. lucidum* in one-year-old tissue culture plantlets (generated through embryo culture) of coconut and arecanut. The roots of these plantlets were soaked in the G9 mycelial suspension for 30 min before being transplanted onto Y3 media for further growth and observation.

Method validation

Mycelial inoculation in coconut and arecanut seedlings

In the mycelium inoculation technique employed, symptoms indicative of BSR disease emerged rapidly following inoculation with *G. lucidum*. In coconut, noticeable symptoms appeared approximately one month post-inoculation, whereas in arecanut, symptoms developed significantly earlier, becoming evident within just two weeks. This variation in symptom onset highlights the importance of recognizing species-specific timelines, providing valuable insights into disease progression and aiding in the timely evaluation of resistance in both coconut and arecanut seedlings.

The symptomatology observed in coconut and arecanut seedlings was remarkably similar. The initial stage involved leaf yellowing, followed by leaf necrosis and crown deterioration, ultimately leading to severe wilting symptoms (Fig. 2). Additionally, extensive rotting of the stem and root portions was observed in both seedlings (Fig. 3a, 3b). Longitudinal sections of the stems of inoculated plants revealed distinct browning and rot in the crown region (Fig. 3c, 3d), in stark contrast to healthy plants, which exhibited no such symptoms. Mycelial colonization was prominently observed in many infected seedlings, particularly concentrated in the root region (Fig. 3e).

In our study, the streamlined inoculation process was completed within 50 days for coconut, including four days for the growth of *G. lucidum* culture and an additional 14 days for morphological and molecular characterization. In the case of arecanut, the entire process required only 30 days. Notably, this technique proved significantly faster than the standard methods, such as the Rubber Wood Block (RWB) and sorghum seed inoculation methods described by Karthikeyan et al[8], which typically require approximately six months for disease assessment. Furthermore, no standardized pathogenicity or inoculation methods have been established for arecanut to date.

Inoculation to coconut and arecanut tissue culture plantlets

Visible symptoms became evident in the tissue culture plantlets of coconut and arecanut, aged one year, just one week after the inoculation process. This rapid onset of symptoms underscores the efficiency of the inoculation method and highlights the susceptibility of the plantlets to *G. lucidum*. The observed symptoms include yellowing, necrosis leading to final wilting of the entire plantlets. Additionally, abundant mycelial colonization around the root and stem areas was also noticed in all the plantlets.

Evaluation and assessment of disease

Disease evaluation was conducted after four weeks of incubation for coconut, while for arecanut, it began at two weeks. Disease incidence (%) was calculated for both set of experiments separately.

$$\text{Disease incidence}(\%) = \frac{\text{No. of seedlings infected} \times 100}{\text{Total no. of seedlings}}$$

Disease scoring was measured both by evaluating external yellowing/wilting symptoms and internal rotting symptoms. The following formula was used for scoring the disease severity of external symptoms:

$$\text{Severity of foliar symptoms}(\%)(\text{Sariah and Zakaria, } 2000) = \frac{(a \times 1) + (b \times 0.5) \times 100}{C}$$

a = number of desiccated (browned/wilted leaves).
b = number of yellowing leaves.
c = total number of leaves.

Disease scoring for internal rotting was measured on a scale of 0-4 according to the observed percentage of rotting symptoms of the disease, 0 = Healthy: no internal rot, 1 = 20% rotting of tissues, 2 = 20–50% rotting of tissues, 3 = >50% rotting of tissues, 4 = >90% rotting of tissues [6].

Disease incidence and severity were assessed separately for seedlings affected by BSR disease, taking into accounts both the mortality and rotting of the seedlings. After four weeks of inoculation, a disease incidence of 66% was recorded in coconut seedlings, while in the case of arecanut seedlings, an 82% disease incidence was observed just two weeks post-inoculation.

Percent severity of foliar symptoms was 56.75% for coconut and 71.83% for arecanut seedlings when compared with 50 replications. On examination of internal rotting symptoms, most of the coconut and arecanut symptomatic seedlings gave an average score of 2.1 and 3.02 after four and two weeks after inoculation (DAI) respectively.

Verification of G. lucidum

To confirm that the observed disease symptoms in coconut and arecanut seedlings, as well as tissue culture plantlets, were caused by *G. lucidum*, pathogenic isolates were re-isolated from the infected roots and collar regions using PDA and GSM. Molecular tests (PCR) were then performed to identify the isolated fungus. Roots from both healthy (control) and diseased coconut and arecanut

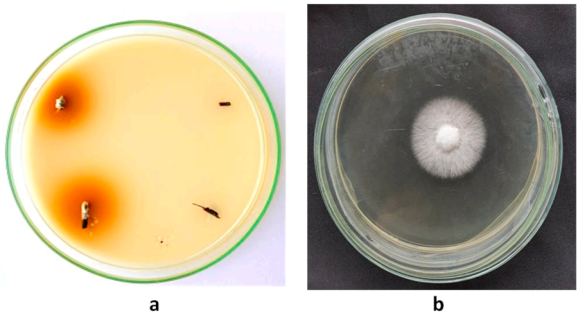

Fig. 4. *Re*-isolation of *G. lucidum* from roots of infected seedlings (a) Plant roots inoculated on Ganoderma Selective Medium (GSM) from infected (reddish coloration) and healthy seedling roots, (b) Pure culture of *G.lucidum* isolated from the infected roots.

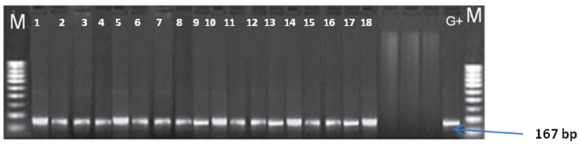

Fig. 5. Pathogenicity confirmation by Polymerase chain reaction from *G. lucidum* inoculated coconut and arecanut seedlings and tissue culture plantlets. Lane M: DNA ladder; Lane 1–8: coconut seedlings; Lane 9–16: arecanut seedlings; Lane 17 & 18: tissue culture coconut and arecanut seedlings; Lane G+: Positive DNA.

seedlings, as well as tissue culture plantlets, were cut, washed with water, and surface-disinfected using 1% (v/v) sodium hypochlorite solution for five minutes, followed by rinsing with sterile distilled water. Each root piece was then cultured on GSM (Chong et al., 2010) and incubated at 30°C for one week.

After the incubation period, fungal growth around the infected roots was observed. Consistent re-isolation of the fungus from infected roots revealed that the morphological characteristics of the fungal isolates closely resembled those of *G. lucidum* in both coconut and arecanut seedlings (Fig. 4).

These fungal colonies were re-isolated and grown on PDA for six days. Mycelia that grew on the media were collected and genomic DNA was extracted using DNeasy Plant Mini Kit (Qiagen). Fungal identification was performed by analyzing DNA sequences using G1 (5′-TCCGTAGGTGAACCTGCGG-3′) and G2 (5′-TCCTCCGCTTATTGATATGC-3′) primer pairs designed from Internal Transcriber Spacer (ITS) region 1 of rDNA of *Ganoderma* sp[7]. The thermo cycler was programmed as, 5 min preheating at 95°C followed by 48 cycles consisting of denaturation at 94°C for 40 s, annealing at 52°C for 40 s and extension at 72°C for 45 s with a final 12 min extension at 72°C. The desired amplicon (Fig. 5) were then extracted and sequenced (Applied Biosystems® 3130 Genetic Analyzer, Thermo Scientific) and verified. Furthermore, sequencing of the ITS regions of these isolates revealed a high degree of similarity with the published sequences of *G. lucidum*, registering 100% nucleotide similarity (supplementary material).

Overall, this study introduces a new inoculation method that quantifies inoculum concentration, providing a precise tool for selecting coconut and arecanut cultivars resistant to *Ganoderma*. Beyond its application in breeding, this method also supports fungicide efficacy testing and enables rapid, reproducible *in planta* assays for disease tolerance and gene function analysis, contributing to improved *Ganoderma* management in plantations. This inoculation method is a cost-effective alternative to traditional field screening, reducing expenses related to land use, labor, and prolonged trials. Using a simple nutrient medium with 0.002% Tween® 20 makes it economically viable for research institutions and farmers. Rapid screening accelerates breeding programs by identifying resistant cultivars early, enhancing yield stability and economic sustainability. Environmentally, it minimizes pathogen spread and soil contamination by confining inoculation to a controlled setting. The biodegradable Tween® 20 reduces reliance on harmful chemicals, promoting sustainable disease management in coconut and arecanut farming.

Limitations

The age of the seedling is a critical factor in the development of disease symptoms. As seedlings mature, the latency period for the onset of visible symptoms extends, with older seedlings taking longer to manifest detectable signs of infection or disease. This delayed symptom expression in mature seedlings is influenced by their enhanced physiological defenses and structural development.

CRediT author statement

Daliyamol: Conceptualization, experimentation, data analysis, writing, reviewing and editing. **Amruthalekhmi M:** Conceptualization. **Greena KK:** Experimentation and methodology. **Prathibha VH**: Experimentation, **Rajesh MK**: Experimentation . **Divakar Y**: Experimentation. **Vinayaka Hegde:** Reveiwing and editing.

Data availability

The DNA sequences of *G. lucidum* have been deposited in the NCBI database. Additional data from this study are available upon request from the corresponding author.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the Coconut Development Board (CDB) through the project 'Diversity analysis of Ganoderma spp. infecting coconut and their eco-friendly management' implemented at ICAR-CPCRI

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2026.103969.

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