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Identification, characterization and cultivation of a wild strain of *Ganoderma australe* from Sikkim, India

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ABSTRACT

Pure culture of *Ganoderma* species was isolated from wild forests of the eastern Himalaya, Sikkim, India. Macroscopic, microscopic, and molecular analysis, including ITS sequencing, confirmed the identity of the strain as *Ganoderma australe* (Fr.) Pat. The species are reported to have antimicrobial properties and potential bioactive compounds. The pure culture was used for spawn production in two different substrates (maize and wheat). For substrate preparation, different woods sawdust and rice straw was used as the base materials supplemented with cornmeal and wheat bran. Fructification was observed in all the substrate combination bags. *G. australe* has not been reported from the eastern Himalayan region of Sikkim. The present findings underscore the rich fungal biodiversity of Sikkim and also provide valuable insights into effective in vitro cultivation strategies of potential medicinal mushrooms.

Keywords: *Ganoderma australe*, medicinal mushrooms, Sikkim Himalaya, spawn production, fructification

Among the diverse array of medicinal mushrooms found in the world over, *Ganoderma* spp. have garnered considerable attention due to their therapeutic potential and long-standing use in traditional medicine, especially in Asian countries, where they are known as “Lingzhi” in China and “Reishi” in Japan (Wu *et al*., 2020; Zhou and Dai, 2012). The bioactive compounds extracted from these fungi, includes triterpenoids, polysaccharides, and sterols, have been demonstrated to possess a range of therapeutic effects, such as immunomodulatory, anti-inflammatory, and anticancer activities (Hapuarachchi *et al*., 2015; Chen *et al.*, 2012). Previous studies have revealed a remarkable diversity of *Ganoderma* species worldwide (Yuen and Hyde 2002; Richter *et al*. 2015). The bioactive compounds are extractable from either the *Ganoderma* mycelium or fruiting body and represent important components of the expanding *Ganoderma* biotechnology industry (Paterson, 2006). Up to now, 181 species are taxonomically accepted under the genus *Ganoderma*, making it one of the most species-rich genera in Ganodermataceae (Costa Rezende *et al.*, 2020). The ability of these fungi to decompose lignocellulosic materials makes them integral to forest health and ecosystem stability (Schwarze *et al.*, 2000). *Ganoderma* are ecologically indispensable as saprophytes, but some of them are pathogenic and can cause diseases in forest trees; they can cause white rot of hardwoods by decomposing lignin, cellulose, and related polysaccharides (Ding *et al*., 2020).

The biodiverse regions, such as Eastern Himalayas, India, represent a rich and largely

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untapped fungal resource with significant ecological and medicinal potential. The temperate and subtropical climates of the region support a good diversity of macrofungi including the genus *Ganoderma* (Wangdi *et al.*, 2019; 2021). Therefore, germplasm collection and characterization from wild is a critical step in the study of their biology and potential applications. Pure cultures isolation offer important taxonomic features and can be used in biotechnology and biomedicine (Badalyan *et al.*, 2015). Successful cultivation of *Ganoderma* not only provides a reliable supply of fruiting bodies and mycelium for medicinal use but also offers economic opportunities for small-scale farmers and commercial producers. In recent times, advances in cultivation techniques have led to improved yields and quality of *Ganoderma* products, making them more accessible for both research purposes and commercial applications (Stamets, 2000). Since there is no previous attempt for isolation of *Ganoderma sp.* from Sikkim, the present study aims to explore the diversity, detailing methodologies for their isolation and cultivation, and discussing their ecological and medicinal significance, thereby contributing to the fields of mycology, ecology, and medicinal mushrooms.

MATERIAL AND METHODS

Study area and sample collection

Sikkim is a small mountainous state of India, situated in Eastern Himalaya, between 27°04’46" and 28°07’48" North latitudes and 88°00’58" and 88°55’25" East longitudes in terms of GPS coordinates. Spanning approximately 7096 km, it has an elevation ranging from 300 to 8585 meters. Opportunistic and random samplings were carried out between April to July 2021-2022 for sample collection. The habitat and morphological characteristics of the macrofungi were noted and photographed. Fresh specimens were collected with great care without any damage using sterile tools, and soil debris was removed using a soft brush and placed in sterile plastic bags, labeled with the collection date and location, and brought to the laboratory on the same day for further analysis and culture isolation.

Morphological characterization and identification

Macroscopic and microscopic observations were carefully recorded, laying the foundation for further analysis and identification. Macro-morphological characters such as the type of basidiocarp (laccate or non-laccate and stipitate, sessile, or dimidiate), length and width, concentric zones, margin shape (lobed, rounded/acute), margin colour (brown, white, reddish, etc.) were recorded. Spore mass was collected from fresh mature caps after the field visits. The cap with pore side down is placed on clean black and white papers; the fruit body was covered with clean paper and left overnight. Basidiospores obtained from spore prints of each specimen were examined under a microscope by mounting in a drop of lactophenol drop at 100× magnification. Spore colour, spore surface, tube colour, pore size, shape was recorded. The morphological characters recorded were compared with keys and taxonomic literature specific to *Ganoderma* spp. (Ryvarden, 2000; Smith and Sivasithamparam, 2003a; Gottlieb and Wright, 2000; Lincoff, 1982; Lodge *et al.*, 2004; Kornerup and Wanscher, 1978). A voucher specimen was deposited at Mycology and Plant Pathology, Department of Botany, NBBGC, Tadong.

Isolation pure mycelium culture

A standard tissue culture technique as described by Stamets (2000) was followed to isolate pure mycelial culture. *Ganoderma* basidiocarp freshly collected from the wild were thoroughly rinsed with water to remove dirt and dust; surface sterilized with 70% alcohol, again rinsed with sterile water, and blotted dry with tissue paper. Bits of internal tissues (0.5–1 cm) of basidiocarp were taken with the help of sterilized forceps and then surface sterilized with 3% sodium hypochlorite solution for 2-3 minutes before rinsing in sterile distilled water for 3 times, after which the bits were dried on sterile Whatman filter papers under aseptic conditions in the laminar flow inoculation chamber. Placed the explants on the sterilized Potato Dextrose Agar (PDA) amended with chloramphenicol in the petri plates and incubated at 27±2ºC for 7-10 days. Actively growing mycelia were transferred to fresh PDA media and incubated at 27°C for 10–15 days for pure culture isolation. Morphological features of the isolate (shape, colour, and texture of the colony) and growth rate of the mycelium were recorded.

DNA isolation, sequencing and sequence analysis

Genomic DNA obtained from Ganoderma culture isolate (MF-4) was used for molecular identification; the ITS-rDNA region was amplified using primers ITS-4 and ITS-5. The sequencing PCR was set up with the ABI-BigDye® Terminator v3.1 Cycle Sequencing Kit. The sequence data was aligned with publicly available sequences (NCBI) and analyzed to reach the identity of the culture isolate (Altschul, 1990).

Culture preservation

Mushroom spawn production requires the preservation of strains and genetic traits of axenic cultures. The pure culture discs were made with a sterile cork borer (about 3 mm). Culture discs were placed in 10 mL screw cap tubes with paraffin liquid labelled with the strain Id following the methods of Johnson and Martin (1992) and Nakasone et al. (2004). The tubes were subsequently stored in the NBBGC's Myco-patho laboratory at Tadong, Gangtok, Sikkim, at 4°C for short-term preservation. Molecular identification was carried out at the National Fungal Culture Collection of India (NFCCI) , Pune, Maharashtra. For long-term preservation, the pure culture was deposited at the Aghakar Research Institute's culture collection centre in Pune, India, with the accession number (MF-4-5389).

Spawn Preparation

For the present experiment, three different substrates viz., wheat, paddy, and maize grain were used to make the spawn of the mushroom. The grains were washed 2-3 times to remove any dirt and soaked in water overnight to get rid of any kind of residue. The water was drained and the substrate was boiled for 20-30 minutes to soften grains and surface dried on sterile sheets for half an hour to reduce the moisture content. Finally, 10 g of calcium sulphate (CaCO3) was combined with half kg of each grain, to maintain the pH of the grains to around 7 and for clump-free substrates. Sterilized conical flasks were three-fourths filled with substrates, the flask's mouth was closed with non-absorbent cotton plugs, and autoclaved twice at 121°C for 15 minutes at 15 psi on alternate days. The flasks were cooled for 1 hour, then, with the help of a pre-sterilized cork borer, 5 mm discs of mycelial mat from pure cultures along with a fraction of PDA were inoculated into the substrate grains in sterile conditions and incubated in the dark for 15–20 days at a temperature 23^{o}C – 24^{o}C. During incubation, the inoculated bottles were shaken at regular intervals to promote and ensure uniform growth of the fungal mycelium (Borah *et al.* 2019). The prepared spawn was used for inoculating different compost substrate combinations for fruiting development.

Compost substrate preparation

In the present study, rice straw, sawdust, and corn meal and wheat bran were evaluated in six different combinations, viz., 1. Alder sawdust (250g) + cornmeal

(100g), 2. Alder sawdust (250g) + wheat bran (100g), 3. Oak sawdust (250g) + corn meal (100g), 4. Oak sawdust (250g) + wheat bran (100g); 5. Pine sawdust (250g) + corn meal (100g) + Rice Straw (100g), 6. Pine sawdust (250g) + wheat bran (100g) + Rice Straw (100g). All the substrates were added with 10g calcium sulphate ($CaSO_4$) and calcium chloride (CaCO3) each. For the cultivation trials, the methods described by Borah *et al.*, (2019) and Stamets (2000) were followed with slight modifications. The substrates were soaked overnight, boiled for half an hour and surface dried for half an hour at room temperature. The different substrate combinations were then placed into the half filled polypropylene bags, plugged with non-absorbent cotton, sterilized for 1 hour at 121^{o}C (15 b psl) and allowed to cool for another 1 hour. All the compost mixtures were inoculated with spawns and incubated at 27^{o}C in the dark in sterilized conditions until colonization of substrate is achieved. Once the desired primordial initiation is achieved under weak light conditions, the bags were kept in a ventilated chamber for fruit body development at room temperature (20°-25°C).

RESULTS AND DISCUSSION

Morphological characterization

The polypore mushroom specimens, phenotypically similar to *Ganoderma* spp., were collected from hardwood stumps in temperate forest of East district of Sikkim in the month of June 2022. Basidiome annual, sessile but very indistinct, short, thick lateral stipe present, contracted base, laccate when fresh, becoming dull and cracked with age. Pilus woody, 5-17 cm in width, up to 2 cm thick at the base; upper surface is brown to reddish brown; zonation on the surface is typically fan-shaped or semicircular; plano-convex; applanate; margin is soft, slightly lobate, and concolorous with the pileus. Spore print is brown; lower surface is white cream-coloured; pore surface turns light brown with maturity. Pores circular 4–6 per mm, context up to 0.5 cm thick, dry, woody, brown. Basidiospores are mostly broadly ellipsoid elongated; they are truncated, reddish-brown, and have a double-walled inner endosporium. Their length ranges from 7 to 12 µm, and their width is 5 to 6 µm (Fig 1 b). Based on the above characterization, the specimen was identified as *Ganoderma* sp. (Fig 1 a).

Culture characterization

With the overlapping features and the existence of different morphs for the same taxa, morphology-based taxonomy can occasionally fail to resolve species effectively (Hyde *et al.*, 2016). Through the tissue culture method, isolation of pure culture of *Ganoderma* species collected from Sikkim's temperate forest was established on PDA medium. The isolate MF-4 showed white mycelia colonies fast growing within 3-4 days of incubation, and the texture and density are floccose to velvety white. As it matures, the density of mycelia becomes creamy white and leathery, and colonies grew radially (8.80 mm/day), with fine rays at the margins. The mycelia covered the petri plates 90 mm after 10 days of inoculation. Colonies were continuous and dense in the centre, concentric ring; reverse white initially turns pale yellowish pigmentation with age. Optimal growth is observed at temperatures ranging from 25°C to 30°C, usually forming visible colonies within a week (Fig 1c). Hyphae thick and thin, hyaline, smooth walled, branched, septate, anastomoses, and hyphal loops present, 1.52–3.48 µm wide (Fig 1d).

Molecular identification

In general, it is difficult and subjective to identify *Ganoderma* species solely based on morphological evidence, as their phenotypic traits are sensitive to extrinsic factors, such as illumination, ventilation, and

Fig. 1. Ganoderma species macroscopic and microscopic features (a-d); a. *Ganoderma australe* basidiocarp, b. Spore print of basiodiocarp, c. Pure culture of *G. australe* after 10 days of incubation, d. Hyphae of *G. australe*

humidity (Hapuarachchi *et al.*, 2019a). Therefore, in the present study, the molecular identification of the *Ganoderma* species of Sikkim was confirmed through ITS-rDNA sequencing. NCBI GenBank BLASTn search of ITS-rDNA partial sequence of the *Ganoderma* sp. confirmed the identification of *Ganoderma* species from Sikkim as species complex of the *G. australe* with 99.66% sequence similarity with GenBank accession LC084747.1 and LC084736.1. *Ganoderma australe* is a cosmopolitan white rot fungus of tropical regions (Ryvarden and Johansen 1980; Yamashita *et al.*, 2009). The species is recognized to be a species complex comparable to that of the *Ganoderma lucidum* complex (Martinez *et al.*, 1991). Previously, this species was only known from New Zealand (Buchanan and Wilkie 1995). Recently, a specimen of *G. australe was* isolated from Thailand by Luangharn *et al.,* (2017), southern India by Kaliyaperumal and Kalaichelvan (2008), and Taiwan by Wang *et al*., (2020). Chakraborty and Shivekumar (2021) and Wu *et al*., (2020) reported potential bioactive compounds from *G. australe*. Luangharn *et al*., (2017) reported antimicrobial activities of *G. australe* against gram-positive and gram-negative pathogenic bacteria. Considering the potential of this species, an attempt was made to culture the wild strain of *G. australe* for the purpose of preservation and fructification. This study also reports a new record of *Ganoderma austale (Fr.)* Pat. from Sikkim.

Ack Code- 3494, ITS Sequence (NCBI Sequence ID: LC084736.1)

GATCATTATCGAGTTTTGACTGGGTTGTAGCTGGC
CTTCCGAGGCATCGTGCACGCCCTGCTCATCCAC
TCTACACCTGTGCACTTACTGTGGGTTATGGATYG
CGTGTAAAAGCGCGTCCGTGCCTGCGTCTTA
CCACAAACACTATAAAAGTATCAGAATGTGTAT
TGCGATGTAACGCATCTATATACAACTTTCAGC
AACGGATCTCTTGGCTCTCGCATCGATGA
AGAACGCAGCGAAATGCGATAAGTAATG
TGAATTGCAGAATTCAGTGAATCATCGAATCT
TTGAACGCACCTTGCGCTCCTTGGTATTCCGAG
GAGCATGCCTGTTTGAGTGTCATGAAATC
TTCAACCTACAAGCTTTTTAATAATGGCTTGTTAGGCT
TGGACTTGGAGGCTTGTCGGTCTTTATTGATCGG
CTCCTCTYAAACGCATTAGCTTGGTTCCT
TTGCGGATCGGCTGTCGGTGTGATAATGTC
TACGCCGCGACCGTGACGCGTTTGGCGAGCTTCTAA
TCGTCTCGCTTTTGGGACAACTTTTATGACCTCTGACCTC
AAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCA

Fruiting trials

Spawn production is a crucial step in the cultivation process; in the present study maize grains substrates was found to be the best substrate for spawn production since the whole substrate was covered with mycelium within 10 days of incubation. In wheat grains substrate, mycelium establishment was visible in 10–15 weeks. The paddy grain substrate did not support good mycelium growth. The optimal conditions for mycelia growth on spawn were 25–30 °C (Fig 2a). Thawthong (2014) reported good growth of wood rotting fungi in a variety of lignocellulosic substrates, including straw, sawdust, and rice husk.

The result of developmental stages of basidiocarp or fructifications in all the different compost is depicted in Table 1, Fig 2 (b-f). Overall, it took on an average 2-4 months from mycelium running to fructifications in all the substrate combinations. The moisture content in substrates was maintained at 60% to 70% level with good air ventilation with indirect light to stimulate fruiting initiation and also to prevent CO_2 buildup, which could inhibit fruiting. Currently, the methods adopted for commercial production show a variety of forms of substrates, which mainly include the wood log, short basswood segment, tree stump, sawdust bag, and agricultural by-products, such as cotton seed husk, straw, and corn cob, in the cultivation *Ganoderma* (Zhou, 2017; Stamets, 2000). Luangharn *et al*., (2017) used Para rubber sawdust with organic and inorganic additives as a standard cultivation substrate in Thailand for the cultivation of *G. australe*. However, regarding the choice of what

Table 1. Comparison of different substrate combination for initiation of fruiting.

Compost	Mycelium establishment	Primordia initiation	Basidiocarp formation
Substrate 1- Alder sawdust + Cornmeal	10-15 days	1 months	2-3 months
Substrate 2- Alder sawdust + Wheat bran	10-15 days	1 months	2-3 months
Substrate 3- Oak sawdust + Corn meal	10-15 days	1 months	2-3 months
Substrate 4- Oak sawdust + Wheat bran	10-15 days	1 months	2-3 months
Substrate 5- Pine sawdust + Corn meal + Rice Straw	10-15 days	1 months	2-3 months
Substrate 6- Pine sawdust + Wheat bran + Rice Straw	10-15 days	1 months	2-3 months

Fig. 2. In vitro cultivation of *G. australe* (a-f); a-*G. australe* spawn, b- Substrate compost preparation for fruiting, c- Different substrate combination in polybag, mycelium establishment, d- Primodial stage, e-f- Fruiting bodies and brown spore mass

kind of method or raw materials to use, the basic process for the production of fruiting bodies is roughly the same.

CONCLUSION

Identification, characterization and cultivation of *Ganoderma australe* (Fr.) Pat as a previously unrecorded species in the region emphasizes the need for continued exploration and conservation efforts in the eastern Himalaya region, ensuring the preservation of these valuable natural resources. Moreover, *Ganoderma australe* has been reported to possess various bioactive compounds, such as triterpenoids, polysaccharides, and phenolic compounds, which contribute to its potential medicinal properties. Isolation of pure culture and sustainable cultivation practices are essential to protect the natural habitats while enabling the utilization of *Ganoderma* for scientific and commercial purposes. The outcomes of this study have significant implications for various stakeholders, including the pharmaceutical industry, traditional medicine practitioners, and conservationists. This study is the first attempt to artificially cultivate *G. australe* in Sikkim region of India. Although, the biological efficiency of the mushroom was not very good but we could achieve to get the fruiting in the experimental trial. Further studies are needed to standardize the cultivation technology in order to commercialize this mushroom for the Industry.

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