Biology and Cultivation of *Ganoderma* spp.

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(2011-11-129)

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2013

DECLARATION

I hereby declare that this thesis entitled "**Biology and Cultivation of *Ganoderma* spp.**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other university or society.

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Certified that this thesis entitled "**Biology and Cultivation of *Ganoderma* spp.**" is a record of research work done independently by **Mr. Vineeth V. Varma** (2011-11-129) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

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LIST OF ABBREVIATIONS

%	Per cent
°C	Degree Celsius
BE	Biological efficiency
CB	Carrot broth
cm	Centimetre
dia	Diameter
et al.	And others
Fig	Figure
g	Gram
h	Hours
Kg	Kilogram
l	Litre
M	Molar
mg	Milligram
ml	Millilitre
min	Minute(s)
mm	Millimetre
OMB	Oat meal broth
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
ppm	Parts Per Million
rpm	Revolutions per minute

LIST OF ABBREVIATIONS (Continued)

SMS	Spent mushroom substrate
spp.	Species
viz.,	Namely
wt	Weight

Introduction

1. INTRODUCTION

Ganoderma, a basidiomycete, has been priced for its therapeutic values for centuries particularly in the orient (Paterson, 2006). The genus comprises of a large and diverse complex of fungi, many of which are wood rotters and others that are pathogenic on economically important trees and perennial crops (Sankaran *et al.*, 2005). The genus *Ganoderma* was established by Finnish mycologist Peter Adolf Karsten in 1881 (Karsten, 1881), with *G. lucidum* (Curtis:Fr.) P. Karst from England as the type species.

Most species of *Ganoderma* are pathogenic causing root rot disease on a variety of monocots, dicots and gymnosperms (Seo and Kirk, 2000) which results in the death of affected trees. Although it is considered as a serious pathogen worldwide, some species like *Ganoderma lucidum* known as Reishi in Japan and Ling Zhi in China, has been considered as a symbol of good fortune and prosperity and a drug to cure all ailments (Zhao and Zhang, 1994). Jong and Birmingham (1992) reported its use against gastric ulcer, chronic hepatitis, hypertension, nephritis, asthma, arthritis, bronchitis, insomnia, cancer, diabetes and anorexia. *Ganoderma lucidum* is a popular medicinal mushroom that has been used in traditional Chinese medicine (TCM) in Asian countries over the past two millennia and is believed to preserve human vitality and to promote longevity upon regular consumption (Sliva, 2006; Stanley *et al.*, 2005).

Ganoderma is presently cultivated artificially in over 10 countries with an annual production of 4,300 tonnes (Veena and Pandey, 2006). In India, *Ganoderma* based nutraceuticals are growing very rapidly and was estimated to be about US $20.00 million in 2000-2001 (Thakur, 2005). However, very little is known about the commercial cultivation aspect of this mushroom in India. Successful cultivation of

the mushroom on sawdust, wheat straw and various substrate combinations has been reported by many workers in India and Kerala (Rai, 2003; Tiwari *et al.*, 2004; Veena and Pandey, 2006; Mishra and Singh, 2006; Geetha *et al.*, 2012).

The mushroom has very large climatic diversity and occurs in natural habitat of temperate to tropical regions of the world. It survives in large number of host trees around the globe. It can be cultivated on easily available and cheap natural substrates like sawdust, cereal straw, natural log and combination substrates.

Considering these facts, the present study was conducted with the objective of understanding the biology of *Ganoderma* and to evaluate different materials as potential substrates for the spawn production and artificial cultivation of this medicinal mushroom.

Review of Literature

2. REVIEW OF LITERATURE

2.1. SURVEY AND COLLECTION

Extensive surveys have been conducted the world over to identify and collect *Ganoderma* mushrooms. The purpose of these surveys include taxonomical interests, studying ethnomedicinal and therapeutic value, understanding pathogenicity of the genus, isolation into pure culture and further cultivation by artificial means and for advanced molecular studies to understand the protein profile and to get a knowledge of the active constituents of the mushroom.

At present, more than 250 *Ganoderma* species have been described. (Ryvarden, 1994). This has been possible due to extensive and scientific surveys conducted across different countries and continents.

Moncalvo *et al.* (1995 a) examined several collections and concluded that collections of *Ganoderma lucidum* from Asia and Europe belong to different species based on the analysis of DNA sequences derived from the internal transcribed spacer (ITS) and partial nuclear large subunit ribosomal DNA (LSUnrDNA) regions. After collection and study of several samples, Pegler and Yao (1996) mentioned that the morphology of *G. lucidum* in the orient differs from that of *G. lucidum* found in Britain and throughout Europe in having basidiocarps of a more slender stature. Sudirman and Mujjyati (1997) collected several *Ganoderma* sporocarps from Indonesia and observed that the basidiocarp extracts of seven of the studied species inhibit the growth of *Bacillus subtilis*. Gottlieb and Wright (1999 a, b) conducted a detailed taxonomic study of the subgenera *Ganoderma* and *Elfvingia* from southern parts of South America. Seo and Kirk (2000) conducted surveys and reported that

most species of *Ganoderma* are pathogenic causing root rot disease on a variety of monocots, dicots and gymnosperms. Idris *et al.* (2004) conducted an extensive survey across oil palm plantations in Malaysia to identify *Ganoderma* species responsible for basal stem rot disease. Similarly extensive surveys were carried out by Wu and Zhang (2003) to report three new species of *Ganoderma* from Taiwan. Sankaran *et al.* (2005) recorded *Ganoderma* species from India causing diseases in perennial crops, studied their host range and taxonomy and reported four new host trees of *G. lucidum*. The fruiting bodies of *G. lucidum* were collected from Thrissur district in Kerala by Sheena *et al.* (2005) for studying the therapeutic potential of the alcoholic extract of the fungus. The sporocarps were dried, powdered and defatted using Soxhlet apparatus and then methanolic extracts were prepared which were used for further experiments. Wang *et al.* (2009) collected different species of *Ganoderma* and analysed them for the presence of triterpenoids. According to him, the qualitative profiles of the species indicate utility for separating the taxa. Erkel (2009) surveyed the Kandira/Kocaeli forest in the Marmara region of Turkey to collect different strains of *G. lucidum* and isolated them for cultivation on substrates containing different protein and carbohydrate sources. Nasim *et al.* (2010) collected *G. lucidum* samples from the premises of Punjab University campus as well as places like Changa Manga and Allama Iqbal town in Lahore and recorded the host trees of the mushroom as well as the morphological characters of the basidiocarps. The purpose of collection was for molecular analysis using RAPD technique. Samples of *G.lucidum, G.applanatum* and *G.tsugae* were collected by Jha *et al.* (2011) from Kathmandu valley, Nepal as part of their survey to collect ethnomedicinal macrofungi. Sharma *et al.* (2012) surveyed and collected different species of *Ganoderma* from Chattisgarh for obtaining pure culture and to characterize them morphologically and by using biotechnological tools. The morphological and genetic characterization of 22 strains of *Ganoderma* was done by Badalyan *et al.* (2012) in Bologna, Italy. The cultures were isolated from immature fruiting bodies obtained from Armenia, France, Iran and Italy. Mishra and Singh (2012) surveyed forest areas

adjacent to Ranikhet, Almora, Lansdowne, Mussorie, Pantnagar, Kashipur, Dehradun, Haldwani and Mukteshwar of Uttarakhand state, India, during the rainy season of 2008 and 2009 to collect the fruiting bodies of *Ganoderma*. The samples were isolated using tissue culture technique and were used for artificial cultivation and evaluation of yield parameters at G B Pant Agricultural University. Wang *et al.* (2009) clarified the species identity of *G. lucidum* using morphological and molecular techniques. Purposive sampling surveys were conducted in Kerala for the collection and characterization of *Ganoderma* which causes basal stem rot disease of coconut throughout the state (Yunus, 2012).

The morphological characters recorded were compared with that of *Ganoderma* species described by various authors (Ryvarden and Johansen 1980; Corner 1983; Ryvarden 1994; Moncalvo and Ryvarden 1997; Ryvarden 2000; Kinge and Mih 2011).

2.2. ISOLATION AND PURE CULTURING

Initial successful attempts for the isolation of *Ganoderma* were carried out by Henmi as reported by Mishra (2005). Nobles (1948) isolated the fungus and based on the cultural studies provided an 11-character key pattern and descriptions to 126 wood decaying basidiomycetes including *Ganoderma*. This was followed by Naoi (1970), who isolated the fungus from its spores. Chen and Hu (1995) isolated *Ganoderma* by germinating basidiospores collected at the onset of sporulation period. Chen and Miles (1996) isolated the fungus by using tissue culture technique from the inner portion of the basidiocarp and transferring it aseptically to potato dextrose agar (PDA) plates. Mishra (2005) used PDA and malt extract agar (MEA) for isolating the fungus and to evaluate the mycelial growth of different strains of *Ganoderma* on different solid nutrient media. A strain of *Ganoderma lucidum* was originally isolated by Berovič *et al.* (2003) from the Slovenian forests in 1995, stored in the Microbial Collection of the National Institute of Chemistry, Ljubljana, Slovenia and

cryopreserved. This was later used for the submerged cultivation of the mushroom to analyse its active constituents. Erkel (2009) tissue cultured strains of *G. lucidum* to malt extract agar (MEA) media and further used it for spawn production on wheat grains. Bandaranayake *et al.* (2012) isolated *G. lucidum* by placing the mushroom tissues in PDA medium following the tissue culture procedure and used it as mother culture for further spawn preparation and cultivation.

2.3. CULTURAL CHARACTERISTICS

In addition to basidiocarp morphology, cultural characteristics such as chlamydospore production, growth rate and thermophily have been used to differentiate *Ganoderma* species (Adaskaveg and Gilbertson, 1986, 1989). The culture colony of *Ganoderma* species was reported to be white to pale yellow and even, felty to floccose at the optimum temperature on potato dextrose agar (PDA) and they become more yellowish on exposure to light (Seo, 1987; Adaskaveg and Gilbertson, 1989). The cultures grow at different optimum temperatures depending on the species. *Ganoderma* in culture produces various hyphal structures such as generative hyphae with clamp connections, fibre or skeletal hyphae, stag-horn hyphae, cuticular cells and vesicles and hyphal rosettes as well as chlamydospores (Adaskaveg and Gilbertson, 1989). Chlamydospore production, growth rate and thermophily of the cultures are the most important cultural characters that have been used for distinguishing *Ganoderma* species (Seo and Kirk, 2000).

To examine the preliminary growth patterns of the fungi, many researchers have investigated the colour, texture and growth rate of *Ganoderma* on various solid media. The rate and extent of growth (radii or diameter) has been a useful criterion for comparison of fungi (Lonergan *et al.* 1993). Mycelial growth rate has been shown to be a good means of comparing the growth of different fungal species under various environmental conditions (Bilay *et al.*, 2000; Vidal *et al*., 1997). Sharma and Thakur (2010) reported that the culture develops into thick white flocculent growth on solid

media in about two weeks. The appearance is partly due to the deposition of calcium oxalate crystals on the hyphae.

2.4. PHYSIOLOGICAL STUDIES

2.4.1. Effect of different solid media on the mycelial growth of *Ganoderma*

Venkatarayan (1935) observed that the fungus grows slowly on malt and prune-juice agar. Bilay *et al*. (2000) evaluated the growth rate of thirty mushroom cultures on an array of media and their pH-modified variants and found the commercial malt extract agar (MEA) and potato dextrose agar (PDA) to be the best. Gonzalez- Matute *et al.* (2002) assessed the mycelial growth rate of the fungus in different nutrient amended and pH modified variants of malt yeast agar (MYA). Bajaj *et al.* (2003) reported that *G.applanatum* grew well in potato dextrose agar (PDA) medium. Mishra (2005) evaluated the mycelial growth of *G. lucidum* on four solid media *viz.* MEA, PDA, Oatmeal agar (OMA) and Yeast extract agar (YEA) and reported the best growth in Malt extract agar (7.5mm/ day). Jo *et al.* (2009) screened twelve different media to determine their suitability for the growth of *Ganoderma* and found PDA to be the best among them based on colony diameter and mycelial density. Zutshi and Gupta (2013) studied the growth of *G. lucidum* cultures in six different media and found Malt extract agar (MEA) to be the best among them. Yunus (2012) evaluated different media *viz.* PDA, czapek's dox agar, Richard's agar and soil extract agar media for the growth of *G. lucidum* and reported PDA to be the best among them for the growth of all isolates.

2.4.2. Effect of pH on the mycelial growth of *Ganoderma*

Available literature suggests certain contradictions between workers regarding the effect of pH on the *in vitro* mycelial growth of *Ganoderma*.

Cho *et al.* (1993) reported 5.5-6.0 as the optimum pH range for the growth of *Ganoderma* spp. while Jayasinghe *et al.* (2008) reports it to be in the range of 5-9.

Mishra (2005) evaluated the effect of different pH levels *viz.* 5, 6, 7 and 8 on the *in vitro* mycelial growth of *G. lucidum* in MEA medium and reported that an acidic pH range of 5-6 is best suited for its growth. Jo *et al.* (2009) screened the pH range from 4-9 to observe the growth of *G. applanatum* cultures on PDA and found out that the pH range of 6-9 was suited for its best growth. Yunus (2012) reported that a neutral to acidic pH range of 5-7 was best suited for the growth of different isolates of *Ganoderma* from Kerala.

2.4.3. Effect of temperature on the mycelial growth of *Ganoderma*

According to Adaskaveg and Gilbertson (1986), *G.lucidum* has an optimum temperature range of 30-34^{0}C with a maximum growth temperature of 37^{0}C. Mayzumi *et al.* (1997) reported 23-34^{0}C as the optimum temperature range for the mycelial growth of *G.lucidum.* Rai (2003) found out that maximum mycelial growth of *Ganoderma* cultures takes place at 30-35^{0}C. Sharma and Thakur (2010) evaluated five different levels of temperature for the effect on radial growth of three species of *Ganoderma* and reported that a temperature range of 30-35^{0}C was most suited for all strains. Similar result was reported by Yunus (2012) who evaluated different temperature conditions for the *in vitro* growth of selected isolates of *G lucidum*.

2.4.4. Effect of light conditions on the mycelial growth of *Ganoderma*

Shin and Seo (1988; 1989) and Seo *et al.* (1995a, b) reported that the growth of *Ganoderma* mycelium was suppressed by light. Wang *et al.* (2012) studied the effect of quality of light on the growth of *Ganoderma* mycelium and concluded that it has much influence on the growth and metabolism of the mycelium and red LED (light emitting diode), blue LED and dark conditions supported its best growth.

2.5. SPAWN PRODUCTION

Mushrooms can be cultivated on sterilized cereal grains but usually grain colonized with mycelium (grain spawn) is used as an inoculum for the bed substrates.

Grain spawn have high inoculum potential derived from the nutrient base which the fungi can utilize for its further growth and fruiting. This process also ensures rapid permeation of the bed substrates thereby excluding the growth of competitors and aiding rapid production of fruiting bodies. The most common form of mushroom seed or spawn is made with sterilized grains, sawdust, dowels or wood chips (Stamets, 2005). A cheap and effective method of spawn production in polypropylene covers was devised by Thapa *et al.* (1978). They used polypropylene bags as an alternative to glass bottles for the production of *Agaricus bisporus* and *Pleurotus sajor-caju* spawn to reduce the cost of cultivation.

Sinden (1934) first introduced the use of grain spawn for mushroom cultivation. He used grains such as wheat, rye, millet etc., cooked them and mixed them with calcium sulphate and calcium carbonate at the rate of 1% and 3% of the substrate weight. Since that, a lot of substrates including grains and sawdust of different species of trees were used worldwide for *Ganoderma* spawn production. Similarly, the moisture and pH of the substrate are also important factors that will determine the mycelial growth on the spawn. Sinden (1934) proposed a moisture content of 40-50% and a pH of 7.5 for the spawn substrate after sterilization. According to Lemke (1971), the spawn grains should contain 50% moisture and the substrate needs to be maintained at a pH of 6.5 to 6.7 for better mycelial colonization. Fungal mat of *G.lucidum* harvested from a 4-7 days old liquid culture in potato dextrose broth (PDB) was used by Chen and Miles (1996) as liquid spawn. The mats were further homogenized, and the macerate was inoculated onto the substrates in bonsai or ikebana containers previously sterilized at 121^{o}C for 20 mins. Gonzalez-Matute *et al.* (2002) used wheat grains amended with $CaCO_3$ and $CaSO_4$ for spawn preparation. Hafiz *et al.* (2007) used a combination of fermented mango sawdust, rice hull and wood chips mixed with 1% calcium carbonate for spawn preparation for the cultivation of the polypore mushroom, *Ganoderma resinaceum*. Sawdust of broad leaved trees like *Mangifera indica, Eucalyptus camaldulensis, Tectona grandis,*

Albizia richardiana, *Bombax ceiba*, *Albizia procera*, *Borasus flabellifer* and mixture of these in combination with wheat bran in the ratio 2:1 mixed with calcium carbonate (0.2%) was used for spawn production of *Ganoderma* spp. in Bangladesh (Hossain *et al.* 2009). Erkel (2009) used sterilized wheat grains inoculated with mycelium from malt extract agar (MEA) slant for spawn production. A combination of substrates like sawdust, rice polish, dolomite and glucose was used in Sri Lanka by Rajapakse *et al.* (2007) and Bandaranayake *et al.* (2012) to produce mother spawn. Sawdust is primarily used in China, Japan and Korea for spawn production of *Ganoderma* spp. (Veena and Pandey, 2010). However, wheat (Rai, 2003; Dadwal and Jamaluddin 2004; Mishra and Singh, 2006; Negi *et al.*, 2008), paddy (Geetha *et al.*, 2012), sorghum (Veena and Pandey 2006), bajra (Sharma and Thakur, 2010) and other grains (Mishra and Singh, 2006) are the substrates used for spawn production of *G.lucidum* in India (Veena and Pandey, 2010).

Cereal grains and sawdust when used for spawn preparation, is amended with materials like calcium carbonate ($CaCO_3$) and gypsum ($CaSO_4$) to prevent clogging as well as to balance pH of the substrates. Kumar *et al.* (1975) suggested the use of calcium carbonate and gypsum in the proportion 1:3 for better growth of grain spawn.

2.6. CULTIVATION

The first culturing of *Ganoderma lucidum* was done in Europe by Constantin and Matruchot in 1898 and the method was perfected by Matruchot in 1908 by culturing it on leaf stacks of different tree species in humid caves (Isabelle, 2006). Later, a technician of Kyoto University Foodstuff Scientific Research Institute, Naoi (1970) used *"Spore Separation Ganoderma Cultivation Method"* to successfully cultivate this fungus. He first achieved the mass production of the fungus by cultivating the fungus in sawdust containing pots. *Ganoderma* is presently cultivated artificially in over ten countries with an annual production of about 4,900-5000 tonnes in 2002 of which 3,800 tonnes were produced in China alone (Lai *et al.*,

2004). Even though the cultivation technology of this medicinal mushroom has advanced in many Asian countries like China, Korea, Taiwan, Japan etc., the cultivation of this mushroom in India is still in its infancy (Veena and Pandey, 2006). Increasing demand for *G.lucidum* and the nutriceutical products based on it has led to investigation into the suitability of agricultural wastes and sawdust as substrates for commercial bag cultivation of the mushroom (Peksen and Yakupoglu, 2009).

Triratana and Chaiprasert (1991) cultivated *G. lucidum* in a substrate combination of pararubber sawdust, rice bran, gypsum and magnesium sulphate. The substrate was packed in polypropylene bags at the rate of 300 g per bag and incubated at 27-32^{o}C. They found that the average yield varied from 6.7-16.9 g/bag. Triratana *et al.* (1991) used sawdust from different trees like *Hevea brasiliensis*, *Dipterocarpus alatus, Pentacme suavis* and *Tectona grandis* mixed with sawdust, rice bran, magnesium sulphate and calcium sulphate for *Ganoderma* cultivation. They recorded the highest biological efficiency in sawdust of *Hevea brasiliensis* supplemented with rice bran (7.5%). Sawdust supplemented with bran substrate containing calcium is commonly used for *Ganoderma* cultivation (Chen and Miles, 1996). According to Riu *et al.*, 1997 and Stamets (2000), it is normally cultivated on solid substrates or other lignocellulosic materials such as straw, sawdust and log. A successful artificial cultivation of *Ganoderma lucidum* has been reported on most broad-leaved hardwood trees and commonly used species include oak, pecan, elder, choke, cherry and plum (Chen and Chao, 1997 and Chen, 1999). Chen (1999) reported that rice bran or wheat bran addition was essential for the successful cultivation of *G. lucidum*. Stamets (2000) opined that over supplementation with rice bran, beyond 15% of the dry mass of the substrate, inhibits sporocarp development. Moreover, *Ganoderma* species can be cultivated on the sawdust obtained from different kinds of trees described by Olei (2003) and Wasser (2005). Wasser (2005) mentioned that current methods most widely adopted for commercial *Ganoderma* cultivation are the wood log, short wood segment, tree stump, sawdust bag and bottle procedures. Veena and Pandey (2006)

observed poor yield in *Ganoderma* beds that were supplemented with rice bran beyond 20% and also determined the preferred pH range of 4.0-6.5 and an optimum temperature of 30^{o}C for mycelial growth in the mushroom beds of local *Ganoderma* isolates.

Chiu *et al.* (2000) used sawdust compost with sawdust, lime, sucrose and wheat bran to cultivate *G. lucidum*. Bajaj *et al.* (2003) reported wheat straw supplemented with wheat bran to be the best medium for cultivating *G. applanatum*. Gonzalez-Matute *et al.* (2002) reported that sunflower seed hull can be used as main energy and nutritional source in the formulation of a substrate for cultivation of *G. lucidum* in synthetic logs with an acceptable mushroom production rate, and the addition of 5% malt to sunflower seed hulls significantly improved the mushroom productivity. Poplar sawdust fortified with wheat bran, calcium sulphate and calcium carbonate was used by Rai (2003) for the cultivation of *G. lucidum*. He observed that the total growth took about four months for completion and on an average the yields ranged between 10-15% biological efficiency. Veena and Pandey (2006) evaluated the growth of *G. lucidum* in ten different substrate combinations based on sawdust and wood chips and found that the yield was higher on sawdust based combinations compared to wood chips based formulations and rice bran gave better yield compared to wheat bran. Mishra and Singh (2006) evaluated twenty substrate combinations comprising of sawdust, wheat bran, rice bran, coir pith, wheat straw and mustard straw for the cultivation of *G. lucidum* and found that among the substrates used, wheat straw + 5% rice bran produced the highest yield. Evaluation of yield potential of *G. lucidum* on saw dusts of *Alnus nepalensis, Aesculus indica, Toona ciliata and Quercus leucotrichophora* was carried out at Uttarakhand by Negi *et al.* (2008). They reported a combination of *Alnus* sawdust and rice bran in the ratio 9:1 to be the most suitable substrate for the cultivation of *G. lucidum*. Suitability of tea waste for the cultivation of *G. lucidum* was investigated by Peksen and Yakupoglu (2009) and it was concluded that tea waste can be utilised as a supplement for substrate preparation

in its cultivation. Erkel (2009) investigated the effect of three kinds of sawdusts *viz.*, poplar, oak and beech, and brans of wheat, rice and corn on the yield of *G. lucidum*. The highest yield and biological efficiency were obtained from oak sawdust compared to other sawdusts and also from wheat bran compared to other supplements. Attempt to cultivate different strains of *Ganoderma* were carried out by Sharma and Thakur (2010) on substrates based on wheat straw, paddy straw, sunflower straw, sawdust and sugarcane bagasse. They reported the combination of wheat straw with sawdust to be the best for *Ganoderma* cultivation. Paddy straw based substrate formulations were used for cultivating *G. lucidum* in India (Veena and Pandey, 2011) and they reported a biological efficiency ranging from 25.7% to 29.9%. The feasibility of cultivation of *G. lucidum* in Kerala was assessed by Geetha *et al.* (2012) using locally available cheap substrates like paddy chaff, rubber sawdust, sugarcane bagasse and paddy straw. They reported the highest biological efficiency of 15% in the substrate combination of rice chaff (80%) + rice bran (20%) + $CaCO_3$ (2%) + sugar (1%) and water (55-60%).

2.7. PROXIMATE CONSTITUENT ANALYSIS

Tseng *et al.* (1984) analysed the chemical compositions of 14-day old dried mycelia of *G. lucidum* and reported the total carbohydrates as 75.0%, lipids as 4.6%, soluble proteins as 8.3% and total ash as 3.62%. Hafiz *et al.* (2007) estimated the proximate constituents of the polypore mushroom *Ganoderma resinaceum* on wet weight basis and reported the moisture content as 60.8%, protein content as 4.3%, fat as 0.8%, ash as 0.7%, crude fibre as 11.3% and carbohydrate as 22.1%.

2.8. SUBMERGED CULTURE PRODUCTION OF MUSHROOMS

Ganoderma lucidum being rare in nature, the amount of wild mushrooms is not sufficient for commercial exploitation. Therefore, its cultivation on solid substrates, stationary liquid medium, or by submerged cultivation has become

essential to meet the increasing demands in the international markets (Berovič *et al.*, 2003).

A successful artificial production of *Ganoderma applanatum* by submerged cultivation in liquid media has been reported by Kohlmünzer *et al.* (1989). Yang and Liau (1998) suggested that optimal initial pH for mycelial growth of *G.lucidum* depended on the culture medium and reported an ideal pH of 4 to be the best in a glucose-ammonium chloride medium. It was reported that the growth rate of *Ganoderma* in shaker flask cultures decreased significantly above and below the optimum temperature range of 30-35^0 C. The advantage of submerged cultivation over traditional basidiocarp cultivation is the reduction in the time spent to obtain the product (Wagner *et al.*, 2003). The production of basidiocarp takes at least three to five months, while reasonable amounts of biomass can be obtained by submerged cultivation within two to three weeks (Tang and Zhong, 2002). Tang and Zhong (2002) reported a yield of 22.1 g/L of biomass from the submerged cultivation of *Ganoderma*.

Materials and Methods

3. MATERIALS AND METHODS

3.1. SURVEY AND COLLECTION

Forest and homestead areas in Kerala, especially around Thiruvananthapuram district, were surveyed during the rainy season of 2011 and 2012 to collect the fruiting bodies of *Ganoderma*. Sporocarps belonging to different stages of growth were collected as far as possible. The collected samples were numbered, and the parameters like location, date of collection, host tree, condition of the host, soil type, vegetation, morphology and age of the sporocarps were noted. The collected fruiting bodies were placed in brown paper covers, brought to the laboratory and examined. These fruiting bodies were identified as *Ganoderma* based on morphological characters. The sporocarps brought to the laboratory were classified based on morphology and were described in detail. The dimensions of the fruiting bodies (length, width and height) were measured and the surface texture and margin shape were recorded. The number of pores per square centimeter was also recorded. Sporocarp samples were then preserved for further studies.

Macromorphological features were recorded from fresh material. Characters that were considered included type of basidiomata, basidiocarp appearance, pileus shape, colour and dimensions, surface texture, margin type and characteristics, hymenophore colour and pore density. Microscopic characters like size, shape and colour of basidiospores as well as presence or absence of chlamydospores also were recorded.

3.2. ISOLATION AND PURE CULTURING

The fungus was isolated using standard tissue culture method. The sporocarps were thoroughly cleaned using water and wiped dry using tissue paper. Bits of tissue

about 3 mm^2 in size from the inner portion were cut using a sterile sharp blade. Surface sterilization was done using absolute alcohol for 30 seconds inside a laminar air flow chamber. These bits were placed in a sterile filter paper and dried. The surface sterilized bits were placed in sterile petriplates containing solid potato dextrose agar medium (PDA), four pieces per plate, using a sterile forceps. The plates were incubated for a week at 28^oc to obtain radial mycelial growth of the fungus. The isolated cultures were further purified by hyphal tip method. Circular discs of 5 mm diameter were cut from the mycelial edges and sub cultured to PDA slants for further studies and preservation. The stock culture was preserved by periodic subculturing at fortnightly intervals to PDA slants and by keeping a set of cultures under refrigeration.

3.3.MOLECULAR CHARACTERIZATION OF THE PATHOGEN ISOLATES BY DNA BARCODING USING UNIVERSAL PRIMERS OF ITS

The isolates of the pathogen obtained from different locations were characterized on molecular basis by comparison of the ITS sequences of the isolates. The procedure for molecular characterization was as follows:

3.3.1. DNA isolation using GenElute Plant Genomic DNA Miniprep Kit (Sigma)

The tissue/mycelium (about 50 mg) was transferred to a microcentrifuge tube and ground in 350 µl of lysis solution A and 50µl of lysis solution B using a micro pestle. The mixture was incubated at 65°C for 10 min. with occasional inversion. 130 µl of precipitation solution was added to the mixture, mixed completely by inversion and the sample was placed on ice for 5 min. The sample was centrifuged at 14,000 rpm (Eppendorf Centrifuge 5804R) for 5 min. to pellet the cellular debris, proteins, and polysaccharides. The supernatant was transferred to the GenElute filtration column tube and centrifuged at 14,000 rpm for 1 min. This removed any cellular debris not removed in the previous step. The filtration column

was discarded and 700 µl of binding solution was added directly to the flow through liquid and mixed thoroughly by inversion. 700 µl of this mixture was added into GenElute nucleic acid binding column and centrifuged at 14,000 rpm for 1 min.

The flow through liquid was discarded and the collection tube was retained. The column was returned to the collection tube and the remaining sample was applied to the column. Centrifugation was repeated as above and the flow through liquid and the collection tube were discarded. The binding column was placed into a fresh 2 ml collection tube. 500 µl ethanol-added wash solution was added to the binding column and centrifuged at 14,000 rpm for 1 min. The flow through liquid was discarded and the collection tube was retained. The wash was repeated once more. The binding column was transferred to a new collection tube. Thirty µl of elution solution (pre-warmed to 65°C) was added to the binding column and centrifuged at 14,000 rpm for 1 min. The stock DNA was properly labelled and stored at 4 °C.

3.3.2. Agarose Gel Electrophoresis for DNA Quality check

The quality of the DNA isolated was checked using agarose gel electrophoresis. 1µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0) was added to 5µl of DNA. The samples were loaded to 0.8% agarose gel prepared in 0.5 X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was performed with 0.5 X TBE as electrophoresis buffer at 75 V until bromophenol dye front has migrated to the bottom of the gel. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

3.3.3. PCR Analysis

PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1 X PCR buffer (100 mM Tris HCl , pH - 8.3; 500 mM KCl), 0.2 mM each dNTPs (dATP, dGTP, dCTP and dTTP), 2.5 mM $MgCl_2$, 20 ng DNA, 1 unit of AmpliTaq Gold DNA polymerase enzyme, 0.1 mg/ml BSA and 4% DMSO, 5 pM of forward and reverse primers.

Primers used

Target	**Primer Name**	**Direction**	**Sequence (5' → 3')**	**Reference/Remarks**
ITS	ITS-1F	Forward	TCCGTAGGTGAACCTTGCGG	White *et al*, 1990
	ITS-4R	Reverse	TCCTCCGCTTATTGATATGC	

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

3.3.4. PCR amplification profile

ITS & LSU

95 ^{o}C - 5.00 min

95 ^{o}C - 0.30 min
58 ^{o}C - 0.40 min } 40 cycles
72 ^{o}C - 1.00 min

72 ^{o}C - 5.00 min
4 ^{o}C - ∞

3.3.5. Agarose Gel electrophoresis of PCR products

The PCR products were checked in 1.2% agarose gels prepared in 0.5 X TBE buffer containing 0.5 µg/ml ethidium bromide. 1 µl of 6 X loading dye was mixed with 5 µl of PCR products and was loaded and electrophoresis was performed at 75V power supply with 0.5 X TBE as electrophoresis buffer for about 1-2 hours, until the bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad)

3.3.6. ExoSAP-IT Treatment

ExoSAP-IT (GE Healthcare) consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications.

Five micro litres of PCR product is mixed with 2 µl of ExoSAP-IT and incubated at 37^{o}C for 15 min followed by enzyme inactivation at 80^{o}C for 15 min.

3.3.7. Sequencing using BigDye Terminator v3.1

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufacturer's protocol.

The PCR mix consisted of the following components:

PCR Product (ExoSAP treated) - 10-20 ng

Primer - 3.2 pM (either Forward or Reverse)

Sequencing Mix	- 0.28 µl
5x Reaction buffer	- 1.86 µl
Sterile distilled water	- make up to 10µl

The sequencing PCR temperature profile consisted of a 1st cycle at 96^{o}C for 2 min followed by 30 cycles at 96^{o}C for 30 sec, 50^{o}C for 40 sec and 60^{o}C for 4 min for all the primers.

3.3.8. Post Sequencing PCR Clean up

The master mix I of 10 µl milli Q and 2 µl 125 mM EDTA per reaction was made initially. 12 µl of master mix I was added to each reaction containing 10 µl of reaction contents and was properly mixed. The master mix II of 2 µl of 3 M sodium acetate pH 4.6 and 50 µl of ethanol per reaction was made. 52 µl of this master mix II was added to each reaction. The contents were mixed by inverting and they were incubated at room temperature for 30 min. The contents were centrifuged at 14,000 rpm for 30 min. The supernatant was decanted and 100 µl of 70% ethanol was added to the left behind material. It was again centrifuged at 14,000 rpm for 20 min. The process of decanting and ethanol wash was repeated. Finally, the supernatant was decanted and the pellet was air dried. The cleaned up air dried product was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems).

3.3.9. Sequence Analysis

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 (Drummond *et al.*, 2010).

3.4. CULTURAL CHARACTERISTICS

Five different isolates of *Ganoderma*, namely VG-1, VG-3, VG-7, Vellayani local and DMR-G were grown in potato dextrose agar medium to study the cultural characteristics. The alphabets V and G in the prefix 'VG' denote Vellayani and *Ganoderma* respectively. Initially, the colour, texture, nature of mycelia and the growth rate was recorded. The cultures were incubated for a longer period to understand the temporal variations in terms of colour and morphology. Apart from this, the microscopic characters like spore pattern, chlamydospore production and presence of hyphal structures was studied.

Five mm discs were cut from the actively growing edges of the pure culture of *Ganoderma* isolates and were plated on to PDA medium aseptically. Each culture was replicated five times and observations were taken at an interval of three days. Microscopic slides were prepared from these cultures, mounted using lactophenol cotton blue stain and observed under a Labomed compound microscope at 10x, 45x and 100x magnifications. The cultural characteristics and microscopic observations were recorded.

3.5. PHYSIOLOGICAL STUDIES

3.5.1. Effect of different solid media on the mycelial growth of *Ganoderma*

The experiments were carried out in completely randomized design (CRD). The five isolates were initially grown in PDA medium to observe the colour and nature of mycelial growth. The growth of five isolates of *Ganoderma* was further evaluated in terms of radial mycelial growth (in cm) on five different nutrient media namely potato dextrose agar (PDA), carrot agar (CA), rose bengal agar (RBA), oat meal agar (OMA) and *Ganoderma* extract agar. The compositions of different media are given in appendix (Appendix II).

The media were prepared and transferred to 250 ml conical flasks at the rate of 150 ml per flask and plugged tightly with cotton plugs. The media flasks were sterilized in an autoclave under 15 psi pressure and 121^{o}C for 20 minutes. The sterilized media was melted in a LG MS 2049UW microwave oven, cooled and poured into sterile petri dishes aseptically inside a laminar air flow chamber and allowed to solidify. The poured plates were inoculated the next day using mycelial discs of 5mm diameter, cut from a 7 day old culture plate. The petri dishes were properly labelled and incubated at room temperature (28 ± 2^{o}C) for 1 week. Five replications were maintained for each treatment in completely randomized design and colony diameter, colour and nature of mycelial growth was measured daily for 7 days. Mycelial growth (in cm) was measured as described by Lonergan *et al.* (1993). The measurements were taken at two different points and the average was recorded. Observations were taken till the fungal colony covered full plate.

3.5.2. Effect of pH on the mycelial growth of *Ganoderma*

PDA was used for studying the effect of pH on mycelial growth of *Ganoderma* cultures. The medium was prepared and the pH was adjusted to 6.0, 7.0 and 8.0 by adding 0.1 N hydrochloric acid (HCl) or 0.1 N sodium hydroxide (NaOH). Sterilization was done by autoclaving at 121^{o}C and 15 psi pressure for 20 minutes. After cooling, the medium was poured into petri dishes under aseptic conditions and allowed to solidify. Two cultures Vellayani local and VG-7 were used for the experiment. The culture disc of five mm diameter cut out from a seven day old culture was used for inoculation. The discs were inoculated at the centre of the dishes and sealed with parafilm. The inoculated dishes were incubated at room temperature (28 ± 2^{o}C). Six replications were maintained for each treatment in CRD and colony diameter and nature of mycelial growth were measured daily for 10 days.

3.5.3. Effect of temperature on the mycelial growth of *Ganoderma*

Twenty ml each of PDA was poured into sterile petri dishes under aseptic conditions. Discs of 5mm diameter were cut from the active growing edges of the two *Ganoderma* cultures *viz*. Vellayani local and VG-7. These discs were placed at the centre of the medium and incubated at ambient temperature, cold condition (4^{o}c) and 35 º C. The nature of the growth and radial mycelial growth was measured daily and recorded for 10 days.

3.5.4. Effect of light conditions on the mycelial growth of *Ganoderma*

Twenty ml each of PDA was poured into sterile petri dishes under aseptic conditions. Discs of 5 mm diameter were cut from the active growing edges of the two *Ganoderma* cultures. These discs were placed at the centre of the medium and incubated under artificial light, natural light and dark conditions. The nature of growth and radial mycelial growth were observed daily and recorded for 10 days.

3.6. SPAWN PRODUCTION

Ganoderma isolates Vellayani local and VG-7 were subcultured on petri dishes containing potato dextrose agar (PDA) medium and was used for mother spawn preparation in grains once they formed full fungal colonies in the plates. Two weeks old cultures were used for spawn inoculation. Spawn preparation was carried out using different substrates as described by Sinden (1934).

Four grain substrates and four sawdust based substrates were evaluated for their efficacy in spawn production of the two selected *Ganoderma* isolates Vellayani local and VG-7. The sawdust of coconut was used as hardwood sawdust and the sawdust obtained from rubber was used as softwood sawdust. The grain substrates used in the experiment were paddy, sorghum, horse gram and wheat and the sawdust based substrates used include softwood sawdust, hardwood sawdust, sawdust

combined with rice bran and sawdust combined with wheat bran. In the combination substrates, hardwood sawdust was used. Each treatment was replicated five times.

The substrates were soaked overnight in water before use. The grains were taken out next morning and were boiled just enough to soften them avoiding the seed coat rupture. The excess water was drained off and the grains were spread on a clean silpaulin sheet for drying. The dried grains were mixed thoroughly with calcium carbonate at the rate of 40 g per kilogram grain and filled in polypropylene bags at the rate of 300 g per packet. The sawdust based substrates were taken out from the water, dried and mixed with calcium carbonate in the proportion sawdust 98 %, calcium carbonate 2 % and water 65 % and was filled in polypropylene bags. The filled packets were sterilized in an autoclave at 121^{o}C and 15 psi pressure for 2 h. Next day, the bags were inoculated aseptically inside a laminar air flow chamber with pure culture of *Ganoderma* at the rate of one-third of the agar pieces colonized by the fungal mycelium cut from a fully covered plate using a sterile inoculation needle and incubated at (28 ± 2ºC). The mycelial growth in the spawn packets was observed and recorded. Time taken for complete mycelial colonization of each substrate also was recorded. The spawn thus obtained as mother spawn was used for further spawn production and to raise beds.

3.7. CULTIVATION

Bag system or artificial log method of cultivation was adopted and moisture level of the substrate was maintained at 60-65% by periodic watering. Eight different substrate combinations were used independently for evaluating their efficacy in cultivating the mushroom which included sawdust (90%) + rice bran (10%), sawdust (90%) + wheat bran (10%), wood chips (80%) + rice bran(20%), wood chips (80%) + wheat bran (20%), sawdust (80%) + rice bran (20%), sawdust (80%) + wheat bran

(20%), sawdust (80%) + rice bran (18%) + $CaCO_3$ (1%) + sucrose (1%) and sawdust (78%) + rice bran (20%) + $CaCO_3$ (2%).

The substrates were mixed together and kept covered for three days using a polythene sheet (Geetha *et al.*, 2012). Periodic turning was given to them on a daily basis. On the third day, the substrate was filled into polypropylene bags at the rate of 500 g (wet substrate) per bag. The edges of the bags were then folded and stapled. The substrate bags were sterilized at 121^0C and 15 psi pressure for 2 h. Upon cooling, the substrates were taken inside the laminar air flow chamber and filled into fresh new polypropylene bags along with mother spawn. The substrate and spawn was mixed together thoroughly at the rate of one packet spawn (300 g) per bag. The edges of cylindrical bags filled with spawned substrates were then tightly folded and stapled and kept for incubation in darkness. After complete colonization (3-4 weeks), the mushroom beds were opened and rolled back to expose the upper surface of the spawn run block and were transferred to an air conditioned cropping room. To provide required humidity, the beds were placed on top of bricks kept in a tray filled with water. A layer of gunny bags was spread hung over the top of beds at a height of 1 m (using wooden poles) carefully enough to avoid direct contact with the beds. This gunny bag layer was continuously moistened (5 times per day) to maintain the relative humidity. A temperature of 24^0C and relative humidity of 90-95% was maintained in the cropping room. Observations on spawn run period, days required for primordial initiation, days required for sporocarps formation, total growth period, total number of fruiting bodies, biological efficiency, nature and colour of sporocarps and biological yield were recorded. The harvested fruiting bodies were then oven dried and preserved for analysis. The BE was calculated using the formula:

$$B.E = \frac{Wet\ wt.of\ harvested\ mushrooms}{Wt.of\ substrate}\ X\ 100$$

3.8. ANALYSIS OF PROXIMATE CONSTITUENTS

The harvested and dried sporocarps were powdered for the analysis of proximate constituents like moisture, ash, carbohydrates, crude protein, fat and crude fibre. The moisture content was analysed on wet weight basis whereas the rest of the parameters were analysed on dry weight basis. Both the isolates were separately analysed for its proximate constituents. Standard methods were used for the analysis of all the constituents.

3.8.1. Estimation of moisture content

Ten g (W_1) of the sample was taken in a preweighed crucible and dried in an oven until constant weight was obtained (W_2). The difference between the initial and final weight gives the moisture content, which is converted into percentage.

$$\%\ of\ moisture\ content = \frac{W1-W2}{10} \times 100$$

3.8.2. Estimation of protein

Protein content of *Ganoderma* spp. was estimated by initial assessment of total nitrogen in the sample by Kjeldahl's method.

The estimation is performed in two stages, namely, digestion and distillation. Initially, 0.3 g of dried mushroom powder was transferred to a 100 ml Kjeldahl flask or a long necked digestion tube. 10 ml of concentrated H_2SO_4 and 0.2 to 0.3 g of digestion / catalytic mixture (25 g of K_2SO_4 with 5 g of $CuSO_4$-$5H_2O$ and 0.5 g of metallic selenium powder by grinding in a mortar) was added to this and allowed to stand overnight. This mixture was digested on low flame initially for 10 – 15 min until frothing stops. Then it was digested at high flame for 4 hours till the contents of Kjeldhal flask became clear or pale green or blue. The flask was cooled and the

contents were transferred to 50 ml volumetric flask and the volume was made up to 50 ml with distilled water.

In the second stage, 10 ml of acid digest was transfered to a micro Kjeldahl distillation assembly. 10-15 ml of 40% NaOH was added to it to make the contents distinctly alkaline and the funnel of distillation assembly was washed with small amounts of distilled water 5 to 3 times. Before adding NaOH, boric acid-mixed indicator solution was kept ready at the receiving end of condenser outlet so that outlet is dipped in boric acid (about 20 ml of 2% boric acid-mixed indicator solution taken in conical flask). The distillation was carried out by passing steam into the distillation flask and the colour of boric acid-mixed indicator solution changes from reddish purple to green. The process was continued till all the NH_3 released from distillation of sample was trapped. After the distillation, bluish green coloured ammonia trapped boric acid (ammonium tetraborate) is titrated against 0.01 N H_2SO_4 till colour changes to purple releasing boric acid with formation of $(NH_4)_2SO_4$. A blank without the sample was run to check for contamination and to ensure precision.

Observations and calculations:

Weight of sample	:	0.3 g
Volume of digest	:	50 ml
Aliquot of sample digest taken for distillation	:	10 ml
Normality of H_2SO_4 used in the titration of the distillate:		0.01 N
Volume of standard H_2SO_4 used in sample titration	:	S ml
Volume of standard H_2SO_4 used in blank titration	:	B ml
Titration value	:	S – B

$$\% N = \frac{(S-B) \times N \text{ of } H_2SO_4 \times \text{volume of digest} \times 100 \times 0.014}{\text{Weight of sample} \times \text{aliquot used for distillation}}$$

% Protein = % N x 6.25

3.8.3. Estimation of fat

The extraction of fat was carried out using Soxhlet extraction apparatus (Moore and Stein, 1948).

Five g of the mushroom powder was taken in a thimble. A piece of cotton wool was placed at the top of the thimble to distribute the solvent as it drops on the sample during extraction. The sample packet was placed in the butt tubes of the Soxhlet extraction apparatus. The sample was then extracted with petroleum ether (150 drops/min) for 6 h without interruption by gentle heating. The ether extract was then allowed to cool and was transferred to a preweighed beaker (W1). The ether present in the extract was evaporated on a steam or water bath until no odour of ether remains. The beaker was cooled at room temperature in a dessicator and weighed. The heating was repeated until constant weight was recorded (W2).

Calculation

$$\% \text{ Fat content of the sample} = \frac{(W_2-W_1)}{\text{Weight of sample (g)}} \times 100$$

3.8.4. Estimation of carbohydrates

Total carbohydrate content was estimated by anthrone method (Hedge and Hofreiter, 1962).

Initially, 100 mg of the mushroom powder was weighed and transferred into a boiling tube. The sample was hydrolysed by keeping it in a boiling water bath for three hours with 5 ml of 2.5 N HCl and cooled to room temperature. The cooled mixture was neutralised with solid sodium carbonate until the effervescence ceases. The volume was made up to 100 ml and centrifuged. The supernatant was collected and 0.5 and 1 ml aliquots were taken from this for analysis. The standards were prepared by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard and '0' was taken as the blank. The volume was made up to 1 ml in all the tubes including the sample tubes by adding distilled water. Then 4 ml of anthrone reagent was added to all the tubes. The tubes with the contents were heated for eight minutes in a boiling water bath. It was then cooled rapidly and the green to dark green colour developed was read at 630 nm in a spectrophotometer (systronics UV-VIS spectrophotometer 118). A standard graph was drawn by plotting concentration of the standard on the X-axis versus absorbance on the Y-axis. From the graph the amount of carbohydrate present in the sample was calculated.

Calculation

$$\text{Amount of carbohydrate present in 100 mg of the sample} = \frac{\text{mg of glucose}}{\text{Volume of test sample}} \times 100$$

3.8.5. Estimation of crude fibre

Crude fibre content was estimated by a method described by Maynard (1970) and Misra *et al.* (1975).

Initially, 2 g of powdered sample was extracted with ether or petroleum ether to remove fat (Initial boiling temperature 35 – 38^{o}C and final temperature 52^{o}C). After extraction with ether, 2 g of dried material was boiled with 200 ml of sulphuric acid for 30 min with bumping chips. The extract was then filtered through muslin and washed with boiling water until washings are no longer acidic. It was then boiled with 200 ml of sodium hydroxide solution for 30 min. and filtered through muslin cloth again and washed with 25 ml of boiling 1.25% H_2SO_4, three 50 ml portions of water and 25 ml alcohol. The residue was removed and transferred to a silica crucible (pre weighed crucible, W_1). It was dried for 2 h at 130 ± 2^{o}C. The crucible was then cooled in a dessicator and weighed (W_2). Finally, it was ignited for 30 min. at 600 ± 15^{o}C, cooled in a dessicator and reweighed (W_3).

Calculation

$$\% \text{ of crude fibre} = \frac{\text{Loss in weight}}{\text{Weight of the sample}} \times 100$$

Loss in weight = $(W_2-W_1) – (W_3-W_1)$

3.8.6. Estimation of ash

Three gram sample was transferred to a weighted silica crucible (W_1). It was heated on a Bunsen burner at a low flame and when the substrate charred the crucible

was transferred to a muffle furnace. It was heated at 500 to 550 ºC for about 2 hours till a white ash was obtained. It was then cooled in a dessicator and weighed (W_2). The difference between the two gives the result, which is converted into percentage.

$$\%\ of\ ash = \frac{\mathrm{W2} - \mathrm{W1}}{3} \times 100$$

3.9. SUBMERGED CULTURE PRODUCTION OF MUSHROOMS

Two liquid media namely; carrot broth (CB) and oat meal broth (OMB) were selected for submerged culture production of two strains of *Ganoderma* spp. Vellayani local and VG-7.

100 ml of each media was prepared in 250 ml conical flasks and was sterilized at 121 ^{0}C for 20 minutes in an autoclave. Discs of fungal mycelium were cut from the actively growing edges of the cultures and were inoculated into the sterilized medium at the rate of five discs per conical flask. The flasks were then placed in a shaker at 80 rpm and incubated at 30 ^{0}C. The yield was estimated after two weeks by filtering out the fungal pellets through a preweighed sterile filter paper and weighing them.

Results

4. RESULTS

4.1. SURVEY AND COLLECTION

Surveys were conducted during 2011 and 2012 rainy seasons across Kerala as a result of which 20 samples of *Ganoderma* sporocarps were collected. The samples collected were described with the details recorded (Table 1). The photographs of the mushroom samples were taken (Plates 1 a, b, c, d and e). The morphological characters were recorded in a suitable format devised after referring the morphological descriptions done by several authors (Ryvarden and Johansen 1980; Corner 1983; Ryvarden 1995; Moncalvo and Ryvarden 1997; Ryvarden 2000; Kinge and Mih 2011).

4.1.1. Morphological characteristics of the collected mushrooms

Sample 1- Peroorkkada, Thiruvananthapuram

Basidiomata solitary on dead wood. *Basidiocarp* laccate and sessile. *Pileus* dimensions 2.5 x 3 x 0.5 cm, reddish brown colour, bracket shaped, dry, polished, zoned concentrically. Margin white, thin, rough, irregular and incurved. *Hymenophore* white to creamy. *Pores* crowded, 1600 per cm^2.

Sample 2- Vavvamoola, Thiruvananthapuram

Basidiomata in troops on live tree. *Basidiocarp* laccate and sessile. *Pileus* dimensions 8 x 4.8 x 3.2 cm, reddish brown colour, bracket to horse hoof shaped, dry, polished, irregularly zoned with concentric sulcate zones. Margin white, thin, rough and irregular. *Hymenophore* white to creamy. *Pores* crowded, 2000 per cm^2.

Peroorkkada

Vavvamoola

Ponmudi

Plate 1 a. *Ganoderma* sporocarps collected from different parts of Kerala

Sample 3- Ponmudi, Thiruvananthapuram

Basidiomata solitary on dead wood. *Basidiocarp* non laccate and sessile. *Pileus* dimensions 10.5 x 6 x 2.5 cm, brown to black in colour, flat, bracket shaped, dry, irregularly roughened with concentric zonation. Margin brown, thick, rough, wavy and irregular. *Hymenophore* white to creamy. *Pores* crowded, 2500 per cm^2.

Sample 4 – Nedumangad, Thiruvananthapuram

Basidiomata solitary on dead wood. *Basidiocarp* non laccate and sessile. *Pileus* dimensions 5 x 4 x 2-2.5 cm, brown colour, bracket shaped, dry, irregularly roughened with concentric zonation. Margin brown, thick, rough, wavy and irregular. *Hymenophore* white to creamy. *Pores* crowded, 1600 per cm^2.

Sample 5 – Sreekaryam, Thiruvananthapuram

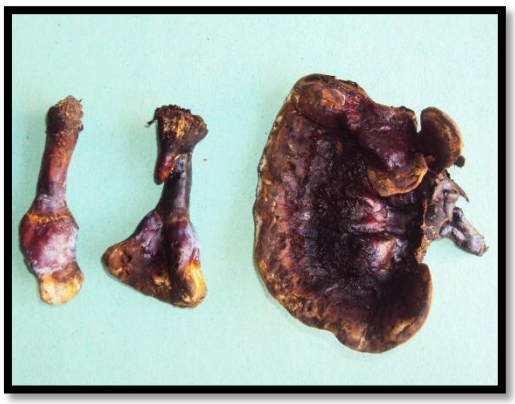
Basidiomata solitary on live tree. *Basidiocarp* brilliantly laccate and stipitate. *Pileus* dimensions 2-3.5 x 0.5-2 x 3-5 cm, cream white colour, subglobose, polished, glossy. *Stipe* dimensions 1.5 x 1 cm, reddish brown, glossy, hard. *Hymenophore* white to creamy. *Pores* crowded, 1700 per cm^2.

Sample 6 – Karyavattom, Thiruvananthapuram

Basidiomata in troops on dead wood. *Basidiocarp* brilliantly laccate and stipitate. *Pileus* dimensions 1-12 x 0.5-7.5 x 4-13 cm, reddish brown colour, slender, cylindrical, tapered or blunt at top, bracket shaped to funnel shaped with lobes, polished, glossy, irregularly roughened. Margin orange to creamy white, thick, rough, wavy, irregular, furrowed and split. *Stipe* dimensions 1.5-4.5 x 0.3- 1 cm, reddish brown, polished, hard. *Hymenophore* white to creamy. *Pores* crowded, 2000 per cm^2.

Nedumangad

Sreekaryam



Karyavattom

Kovilnada

Plate 1 b. *Ganoderma* sporocarps collected from different parts of Kerala

Sample 7 – Kovilnada, Thiruvananthapuram

Basidiomata solitary on live tree. *Basidiocarp* brilliantly laccate and stipitate. *Pileus* dimensions 26 x 14 x 6.8 cm, reddish orange colour, funnel shaped with lobes, dry, polished, glossy, irregularly roughened with radial zonation. Margin creamy, thick, rough, wavy, irregular, furrowed and split. *Stipe* dimensions 2.5-4 x 2-3.5 cm, reddish brown, polished, hard. *Hymenophore* white to creamy. *Pores* crowded, 1750 per cm^2.

Sample 8 – Vavvamoola, Thiruvananthapuram

Basidiomata solitary on live tree. *Basidiocarp* non laccate and sessile. *Pileus* dimensions 16 x 10 x 2 cm, brown colour, bracket to horse hoof shaped, dry, irregularly roughened with concentric zonations and furrows. Margin brown, thick, smooth, wavy and irregular. *Hymenophore* white to creamy. *Pores* crowded, 1800 per cm^2.

Sample 9 – Vavvamoola, Thiruvananthapuram

Basidiomata solitary on live. *Basidiocarp* laccate and stipitate. *Pileus* dimensions 12 x 9 x 5.5 cm, reddish brown colour, flat, funnel shaped, dry, polished, irregularly roughened with concentric zonation. Margin brown, thick, smooth, wavy and irregular. *Stipe* dimensions 1.5 x 1 cm, reddish brown, dry and hard. *Hymenophore* white to creamy. *Pores* crowded, 2000 per cm^2.

Sample 10 – Palode, Thiruvananthapuram

Basidiomata solitary on dead wood. *Basidiocarp* non laccate and sessile. *Pileus* dimensions 15.5 x 8 x 5.5 cm, brown colour, flat, bracket shaped with surface ridges, dry, polished, irregularly roughened with concentric zonation. Margin brown, thick, rough, wavy and irregularly furrowed. *Hymenophore* white to creamy. *Pores* crowded, 1700 per cm^2.

Vavvamoola

Vavvamoola

Palode

Kowdiar

Plate 1 c. *Ganoderma* sporocarps collected from different parts of Kerala

Sample 11 – Kowdiar, Thiruvananthapuram

Basidiomata in troops on dead wood. *Basidiocarp* laccate and stipitate. *Pileus* dimensions 6 x 5 x 2.5 cm, light brown to white colour, bracket to reniform shaped with stipe, moist, polished, concentrically zoned. Margin white, thick, smooth, wavy and regular. *Stipe* dimensions 5.5 x 2 cm, reddish brown, glossy, polished and hard. *Hymenophore* white to creamy. *Pores* crowded, 1400 per cm^2.

Sample 12 –Panniyur, Kannur

Basidiomata solitary on dead wood. *Basidiocarp* non laccate and sessile. *Pileus* dimensions 18 x 13 x 2 cm, brown colour, bracket shaped, dry, polished irregularly roughened with concentric zonation and radial furrows. Margin brown, thick, rough, wavy and irregular. *Hymenophore* white to creamy. *Pores* crowded, 1600 per cm^2.

Sample 13 – Kumarakom, Kottayam

Basidiomata solitary on live tree. *Basidiocarp* non laccate and sessile. *Pileus* dimensions 7 x 4.5 x 2.5 cm, brown colour, bracket shaped, dry, polished, irregularly roughened with concentric zonation and raised surface ridges. Margin brown, thick, rough, wavy and irregular. *Hymenophore* white to creamy. *Pores* crowded, 2000 per cm^2.

Sample 14 – Kowdiar, Thiruvananthapuram

Basidiomata solitary on live tree. *Basidiocarp* laccate and sessile. *Pileus* dimensions 5.5 x 5.5 x 2 cm, yellowish-orange colour, bracket shaped, moist, polished smooth with concentric zonation. Margin white, thick, smooth, wavy and regular. *Hymenophore* white to creamy. *Pores* crowded, 1900 per cm^2.

Panniyur

Kumarakom

Kowdiar

Vanchiyoor

Sreekaryam

Plate 1 d. *Ganoderma* sporocarps collected from different parts of Kerala

Sample 15 – Vanchiyoor, Thiruvananthapuram

Basidiomata solitary on live tree. *Basidiocarp* laccate and sessile. *Pileus* dimensions 8 x 6.5 x 3.5 cm, reddish brown colour, bracket shaped, moist, polished and smooth with no zonation. Margin white, thick, smooth, wavy and regular. *Hymenophore* white to creamy. *Pores* crowded, 2000 per cm^2.

Sample 16 – Arppookkara, Kottayam

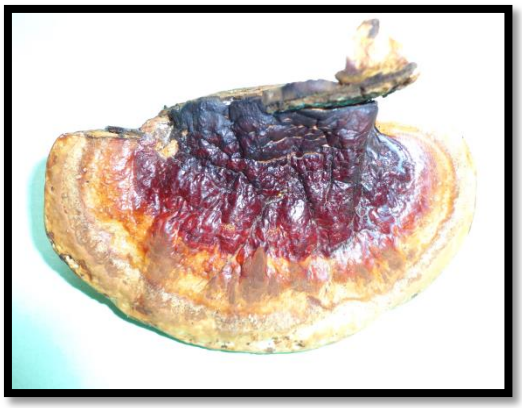
Basidiomata solitary on dead wood. *Basidiocarp* laccate and sessile. *Pileus* dimensions 6 x 4.5 x 2-2.5 cm, yellow colour, subglobose, moist, smooth, polished with concentric zonation. Margin white, thick, smooth, wavy and regular. *Hymenophore* white to creamy. *Pores* crowded, 1200 per cm^2.

Sample 17 – Sreekaryam, Thiruvananthapuram

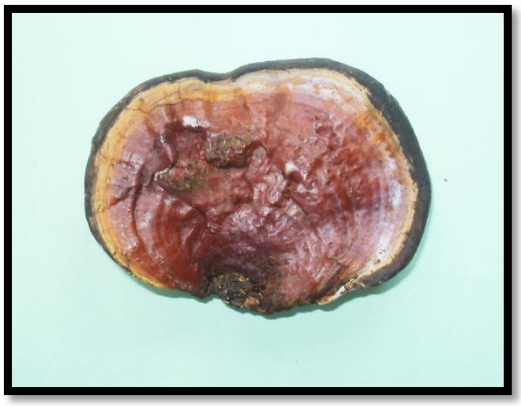
Basidiomata solitary on dead wood. *Basidiocarp* brilliantly laccate and sessile. *Pileus* dimensions 20 x 14 x 4.5 cm, deep red to brown colour, bracket shaped, polished, smooth with concentric zonation and radial furrows. Margin white to orange, thick, rough, wavy and irregular. *Hymenophore* white to creamy. *Pores* crowded, 1600 per cm^2.

Sample 18- Thalassery, Kannur

Basidiomata solitary on dead wood. *Basidiocarp* laccate and sessile. *Pileus* dimensions 6.7 x 4.1 x 3.7 cm, deep red to brown colour, bracket shaped, polished, irregularly roughened with concentric zonation and surface ridges. Margin white, thick, rough, wavy and regular. *Hymenophore* white to creamy. *Pores* less crowded, 900-950 per cm^2.



Kannur



Thrissur

Punnaykkamugal

Arppookkara

Plate 1 e. *Ganoderma* sporocarps collected from different parts of Kerala

Sl.No	Location	Date of Collection	Host Plant	Condition of the host	Colour of the Sporocarp	Age of the Sporocarp
1	Peroorkkada, Thiruvananthapuram	23-10-2011	*Cocos nucifera*	Dead Stump	Reddish brown to white	Tender
2	Vavvamoola, Thiruvananthapuram	31-10-2011	*Artocarpus hirsutus*	Live tree	Reddish brown to white	Mature
3	Ponmudi, Thiruvananthapuram	27-11-2011	*Manilkara* spp.	Dead Stump	Brown to Black	Mature
4	Nedumangad, Thiruvananthapuram	27-11-2011	*Cocos nucifera*	Dead Stump	Brown	Mature
5	Sreekariyam, Thiruvananthapuram	28-11-2011	*Peltophorum ferrugineum*	Live tree	Reddish brown to cream	Tender
6	Karyavattom, Thiruvananthapuram	29-11-2011	*Peltophorum ferrugineum*	Dead Stump	Reddish brown to cream	Mature
7	Kovilnada, Thiruvananthapuram	29-11-2011	*Cocos nucifera*	Live tree	Red, orange and cream	Mature
8	Vavvamoola, Thiruvananthapuram	5-12-2011	*Cocos nucifera*	Live tree	Brown	Mature
9	Vavvamoola, Thiruvananthapuram	12-12-2011	*Cocos nucifera*	Live tree	Reddish brown to cream	Mature

10	Palode, Thiruvananthapuram	17-12-2011	*Areca catechu*	Dead Stump	Brown	Mature
11	Kowdiar, Thiruvananthapuram	03-01-2012	*Areca catechu*	Dead Stump	Reddish brown to cream	Tender
12	Panniyur, Kannur	15-2-2012	*Macaranga hypoleuca*	Dead Stump	Brown	Mature, Old
13	Kumarakom, Kottayam	17-4-2012	*Cocos nucifera*	Live tree	Brown	Mature
14	Kowdiar, Thiruvananthapuram	01-5-2012	*Peltophorum ferrugineum*	Live tree	Yellow and white	Young
15	Vanchiyoor, Thiruvananthapuram	02-5-2012	*Peltophorum ferrugineum*	Live tree	Red and white	Young
16	Arppookkara, Kottayam	21-5-2012	*Cocos nucifera*	Dead stump	Reddish brown	Mature
17	Sreekariyam, Thiruvananthapuram	21-6-2012	*Cocos nucifera*	Dead stump	Deep red to Brown	Mature, old
18	Kannur	16-7-2012	*Anacardium occidentale*	Dead stump	Deep red and white	Mature
19	East fort, Trichur	28-7-2012	*Cocos nucifera*	Dead stump	Reddish brown to orange	Mature
20	Punnaykkamugal, Thiruvananthapuram	09-08-2012	*Cassia fistula*	Dead stump	Deep reddish brown	Mature

Table 1. *Ganoderma* mushrooms collected from different parts of Kerala

Sample 19 – East fort, Trichur

Basidiomata solitary on dead wood. *Basidiocarp* non laccate and sessile. *Pileus* dimensions 8 x 5.5 x 1 cm, orange to reddish brown colour, bracket shaped, dry, polished, irregularly roughened with concentric zonation. Margin white, thick, rough, wavy and irregular. *Hymenophore* white to creamy. *Pores* less crowded, 900-1000 per cm^2.

Sample 20 – Punnaykkamugal, Thiruvananthapuram

Basidiomata solitary on dead wood. *Basidiocarp* brilliantly laccate and sessile. *Pileus* dimensions 31 x 25 x 9.5 cm, deep reddish brown colour, bracket shaped, dry, polished irregularly roughened with concentric zonation. Margin brown, thick, rough, wavy, irregular and incurved. *Hymenophore* white to creamy. *Pores* crowded, 1300 per cm^2.

4.2. ISOLATION AND PURE CULTURING

Three isolates of *Ganoderma* were obtained in pure culture from Sample 1, 2 and 11 and they were named as VG-1, VG-3 and VG-7 respectively. The letters V and G in the prefix denotes 'Vellayani' and '*Ganoderma*'. The pure cultures were sent for identification to Directorate of Mushroom Research, Solan and were deposited to obtain accession numbers DMRO 546, DMRO 547 and DMRO 548 for VG-1, VG-3 and VG-7 respectively. Apart from this; a culture was obtained from Directorate of Mushroom research, Solan and was named DMR-G. Another culture named Vellayani local was procured from Instructional farm, Vellayani. Thus, a total of 5 isolates were used for further studies (Plate 2).

4.3. DNA ISOLATION AND SEQUENCING

Chromatogram from the BLAST analysis of the sequences from different isolates showing the first 100 hits showed that, the isolates were found to have

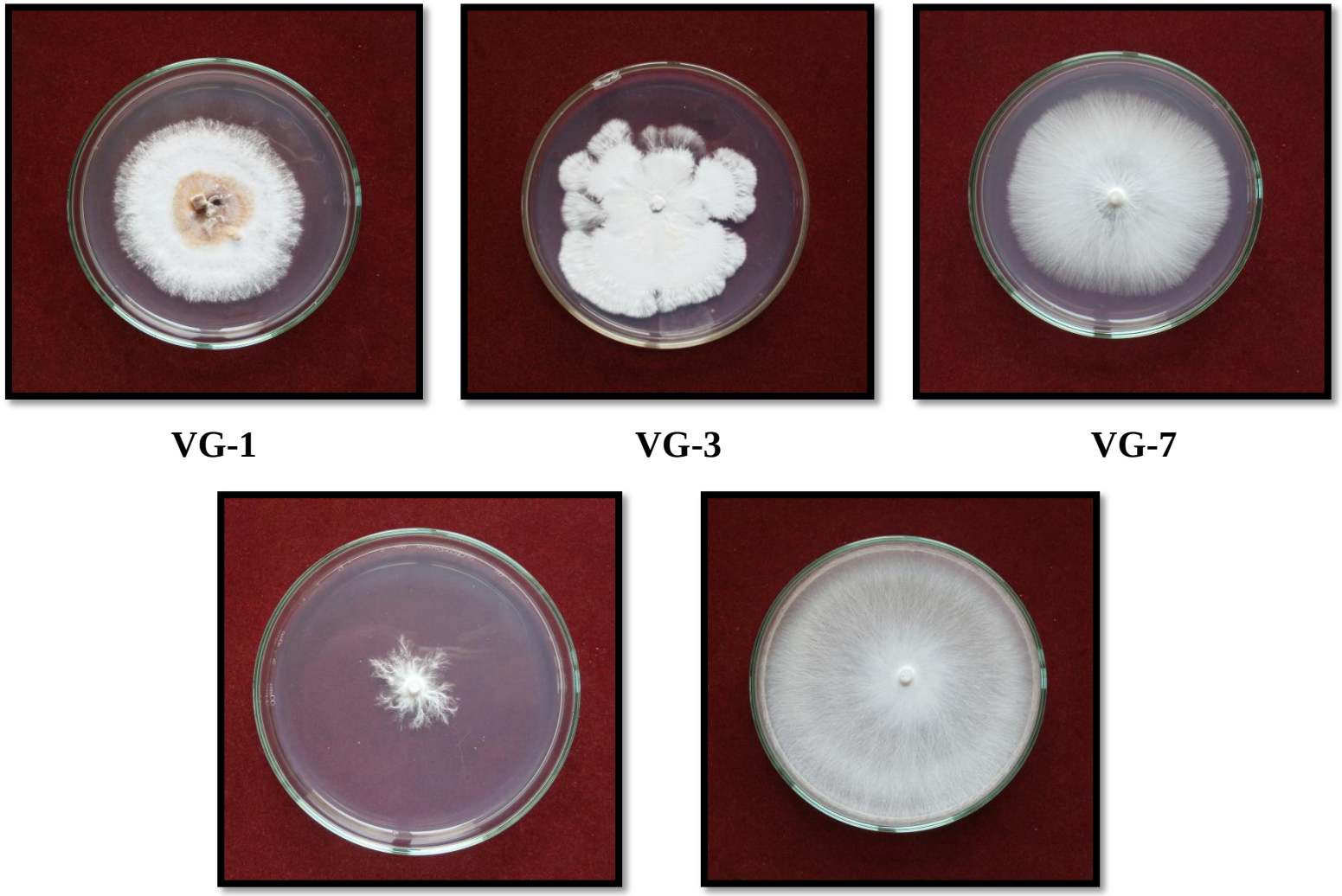


Plate 2. Pure cultures of *Ganoderma* (1 week growth)

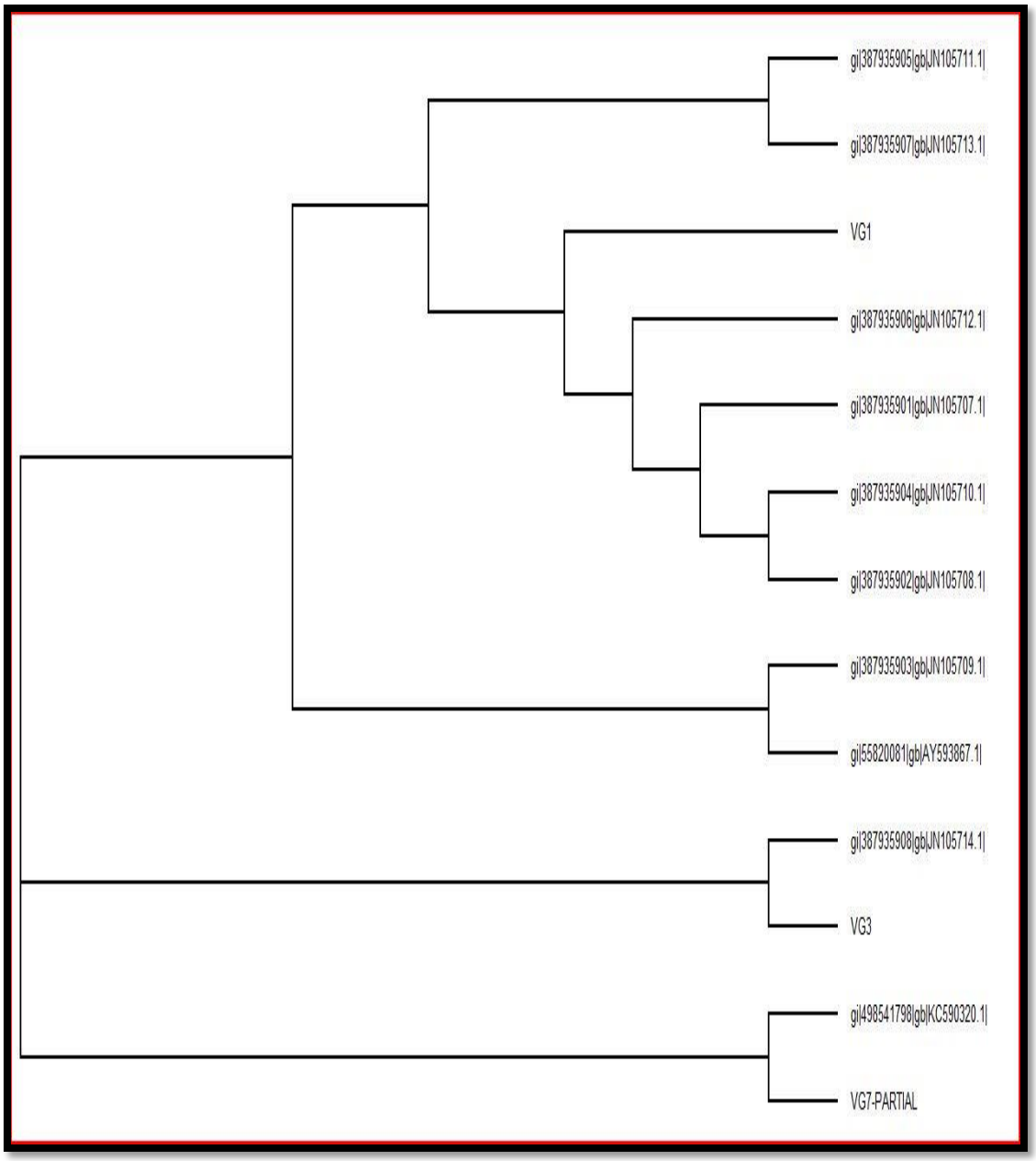


Plate 3. Dendrogram showing the phylogenetic relationship of *Ganoderma* isolates with ten available sequences

similarity with 15 sequences with more than 200 identity keys (red coloured bands). Upon phylogenetic analysis (Clustal X.2.0) using ten available *Ganoderma* sequences (Appendix), the isolate VG-1 was found to have similarity with the sequences gi|387935906|gb|JN105712.1|, gi|387935901|gb|JN105707.1|, gi|387935904|gb|JN105710.1| and gi|387935902|gb|JN105708.1|. The isolate VG-3 was having similarity with the sequence >gi|387935908|gb|JN105714.1|, whereas the isolate VG-7 was observed to be have similarity with the sequence >gi|498541798|gb|KC590320.1| (Plate 3). The sequences are appended (Appendix I).

4.4. CULTURAL CHARACTERISTICS

The cultural characteristics of various *Ganoderma* isolates are given in Table 2. All five isolates initially grew as white mycelia but the mycelial density varied with the cultures. While VG-1, VG-3 and VG-7 grew as thick white flocculent mycelia, Vellayani local and DMR-G isolates developed thin mycelia. The nature of growth also differed considerably. VG-3 and DMR-G initially grew in irregular patterns whereas the other cultures exhibited a circular pattern of growth.

Microscopic characteristics like shape, size and colour of basidiospores and presence or absence of chlamydospores were observed and recorded (Table 3). Pigmentation was observed in three of the cultures after a week of growth due to the production of chlamydospores. The isolates VG-1 and DMR-G developed reddish brown to brown colouration in the culture, while VG-7 produced yellow pigmentation. No pigmentation was observed in Vellayani local and VG-3. Clear and distinguishable zonation was observed in the culture VG-1. The basidium and clamp connections were visible in the mycelia of VG-1 upon microscopic examination (Plates 5-8).

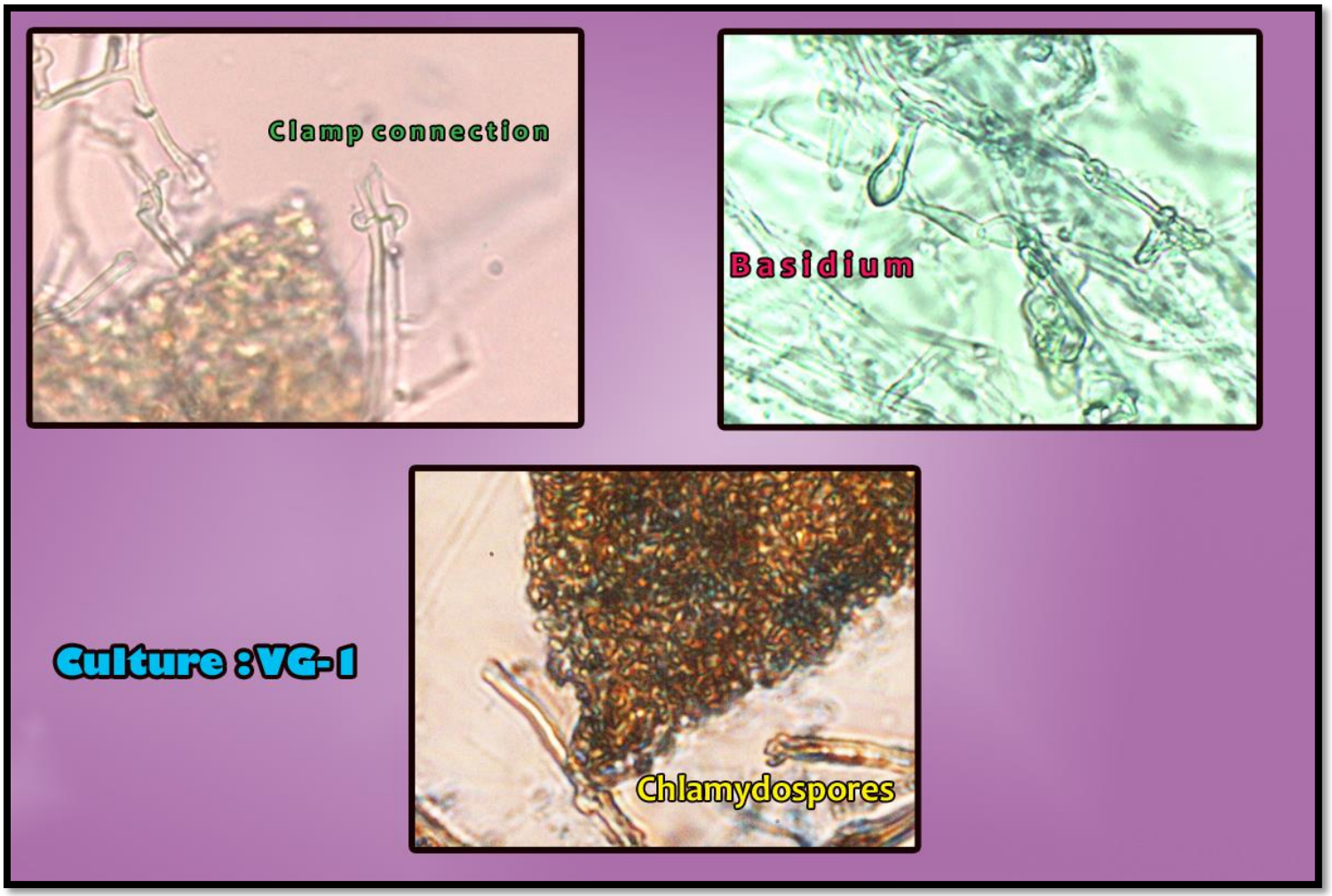


Plate 4. Clamp connection, basidium and chlamydospores of the *Ganoderma* isolate VG-1

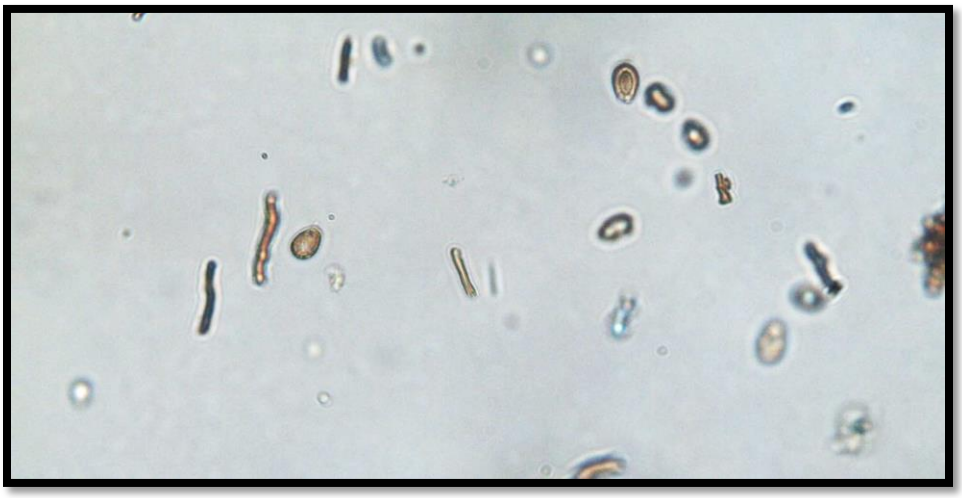


Plate 5 a. Basidiospores of *Ganoderma* isolate VG-1

Plate 5 b. Concentric zonation in culture of *Ganoderma* isolate VG-1

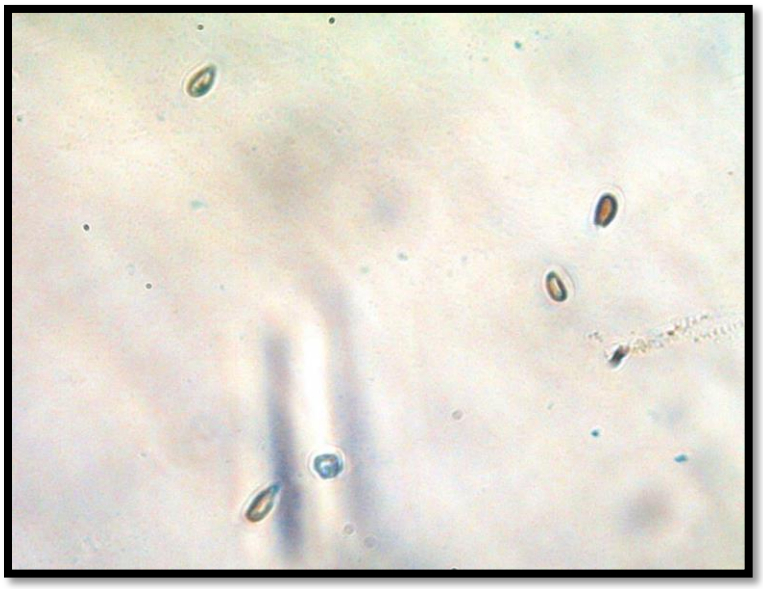


Plate 6 a. Basidiospores of *Ganoderma* isolate VG-3

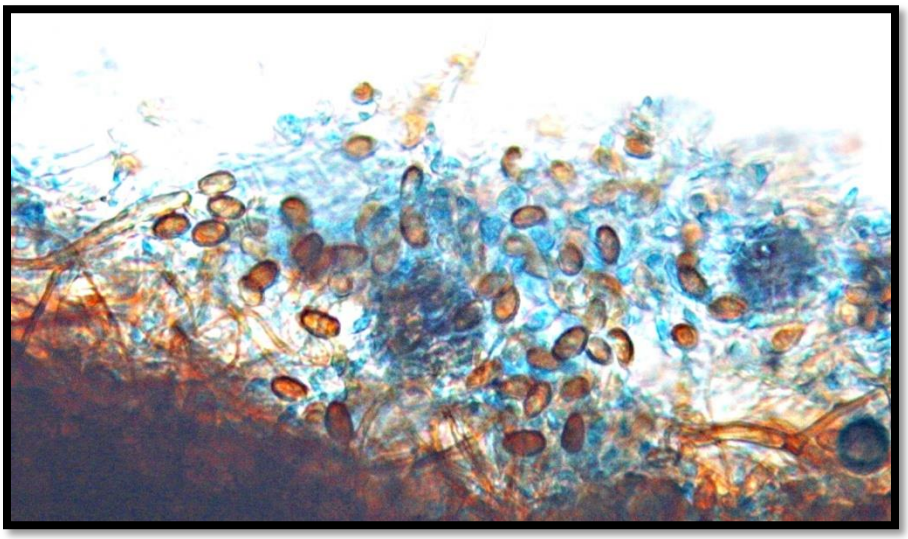


Plate 6 b. Basidiospores of *Ganoderma* isolate VG-7

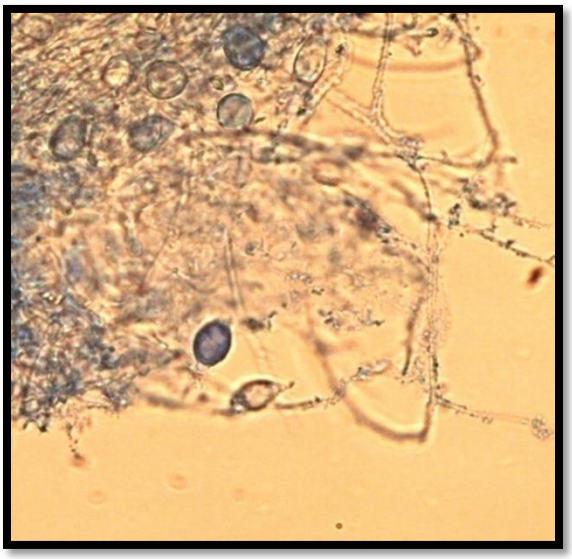


Plate 7 a. Basidiospores of *Ganoderma* isolate Vellayani local

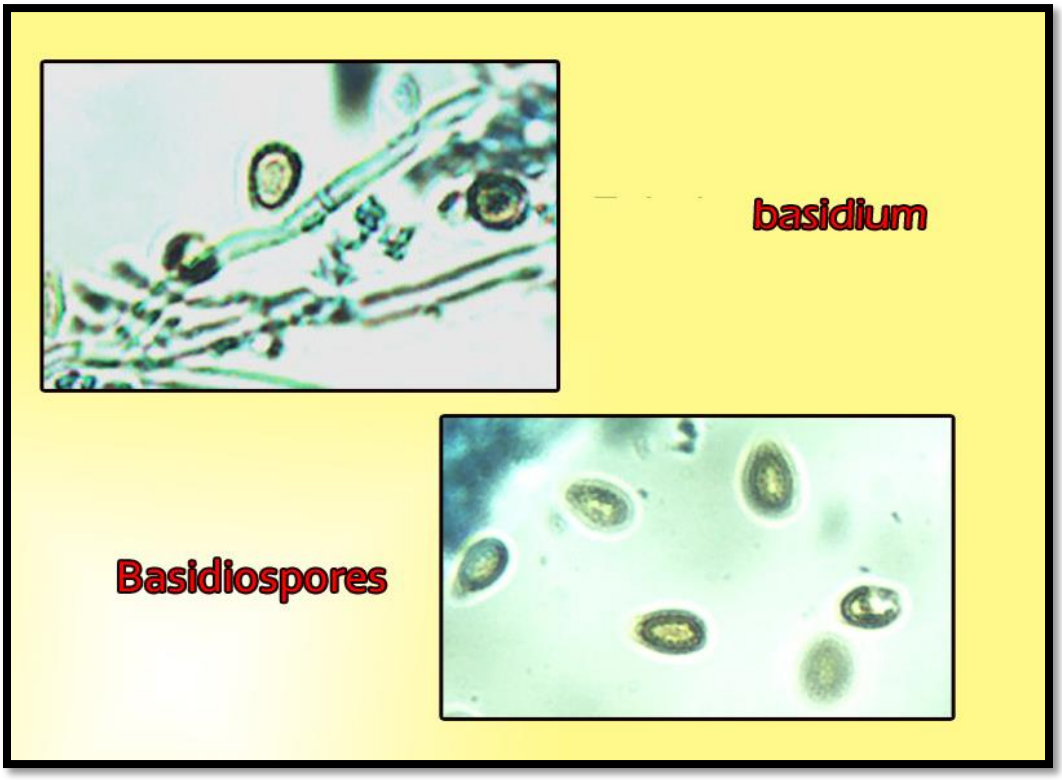


Plate 7 b. Tubular basidium and basidiospores of the *Ganoderma* isolate DMR-G

Plate 8. Fruiting body formation in culture (DMR-Gano)

Isolate	Mycelial colour (initial)	Pigmentation (Chlamydospores)	Concentric zonation	Mycelial characters	Other characters
VG-1	White	Reddish brown	Present owing to diurnal periodicity	Thick and cottony	Papery nature upon ageing
VG-3	White	Yellow	Not distinguishable	Thick and fluffy	Lobed mycelial growth
VG-7	White	Yellow	Absent	Initially thin, later thick	Brown shade at the centre during later stages
Vellayani local	White	Brown when old	Absent	Very thin growth	Fast growing, less dense
DMR-G	White	Brown	Absent	Thin	Sparse and irregular growth. Fruiting body formation in vitro

Table 2. Cultural characteristics of various *Ganoderma* isolates

Cultures	Basidiospores	Chlamydospores
VG-1	Ovoid, brown, 9-11 x 6-8.5µm	Present, clustered, reddish brown
VG-3	Oblong to ovoid, brown, 9-10 x 6-8µm	Absent
VG-7	Oblong to ovoid, dark brown, 10-12 x 5-7µm	Present, golden brown
Vellayani local	Globose to ovoid, light brown, 8-9 x 6.5-8µm	Absent
DMR-G	Ovoid, golden, 9.5-13 x 6-8.5µm	Present, dark brown

Table 3. Microscopic characteristics of various *Ganoderma* isolates

4.5. PHYSIOLOGICAL STUDIES

4.5.1. Effect of different solid media on the mycelial growth of *Ganoderma*

Five different nutrient media were evaluated for their efficacy in supporting the growth of five *Ganoderma* isolates in vitro. The colour and nature of growth of all the isolates were recorded by periodical visual observation. Observations were taken when one of the cultures attained full growth and covered the whole plate (Table 4).

The mycelial growth of five cultures of *Ganoderma* in five different nutrient media was recorded (Plates 9-13). Among the cultures, Vellayani local was the fastest growing one and among the media used, carrot agar (CA) was found to be the best followed by oatmeal agar (OMA) and potato dextrose agar (PDA). The mycelial growth was very low in *Ganoderma* extract agar and overall mycelial growth was poor in rose bengal agar (RBA). PDA was used for further studies since the growth of the best culture Vellayani local was fastest in PDA medium and owing to the ease of its preparation and maintenance in the laboratory.

4.5.2. Effect of pH on the mycelial growth of *Ganoderma*

Three different H^+ ion concentrations (pH) 6, 7 and 8 were evaluated for identifying the best pH condition that supports the growth of selected *Ganoderma* cultures.

Growth of *Ganoderma* isolates Vellayani local and VG-7 in petri dishes was recorded five days after inoculation for determining growth in different pH conditions. The study indicated that slightly acidic pH of 6 was better suited for the growth of VG-7 isolate while Vellayani local preferred a neutral pH of 7 by producing a radial growth of 8.90 cm which was on par with pH 6 (8.76 cm). Among the cultures, Vellayani local grew faster and its growth significantly differed from

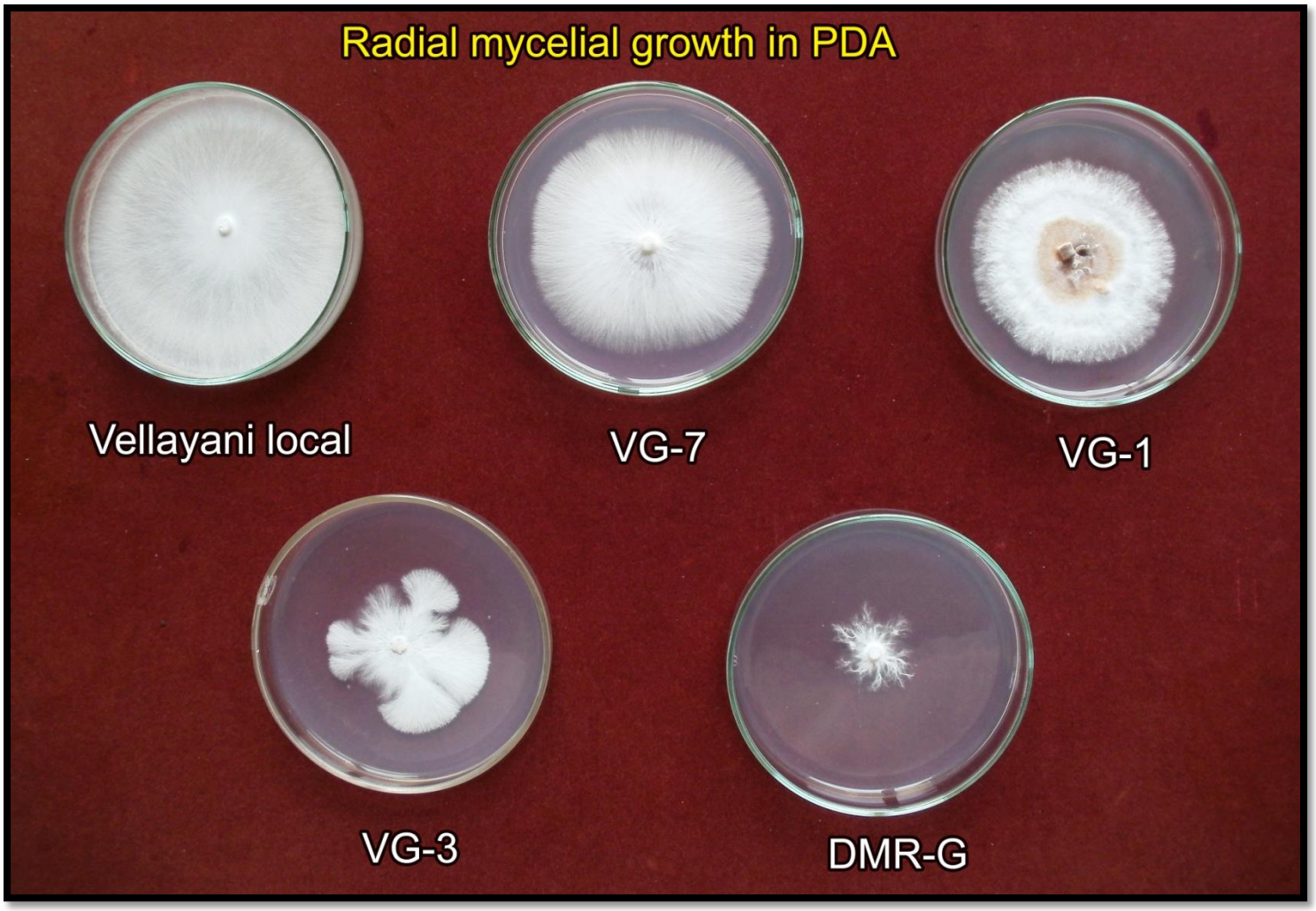


Plate 9. Mycelial growth of 5 isolates in Potato Dextrose Agar medium

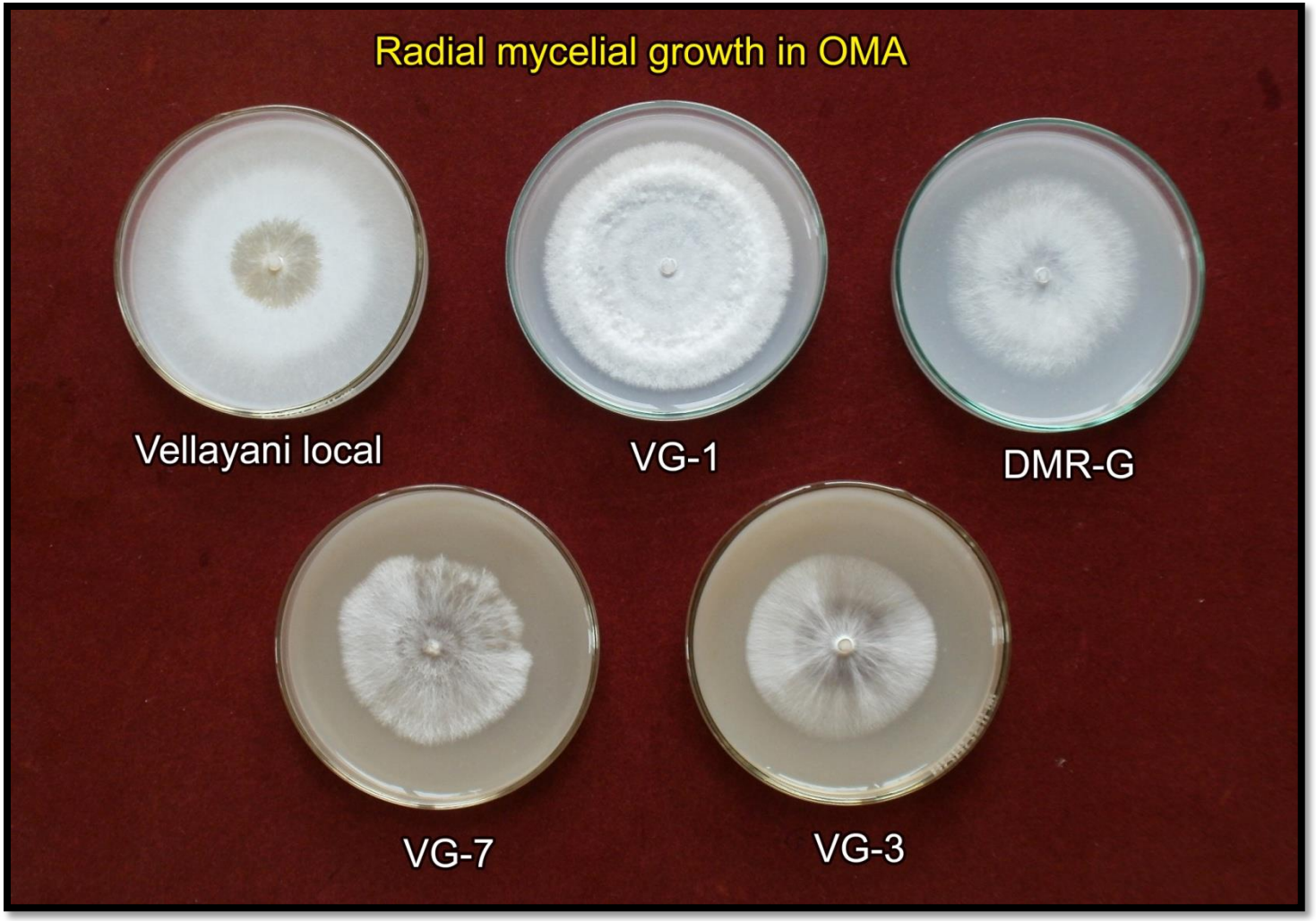


Plate 10. Mycelial growth of 5 isolates in Oatmeal Agar medium

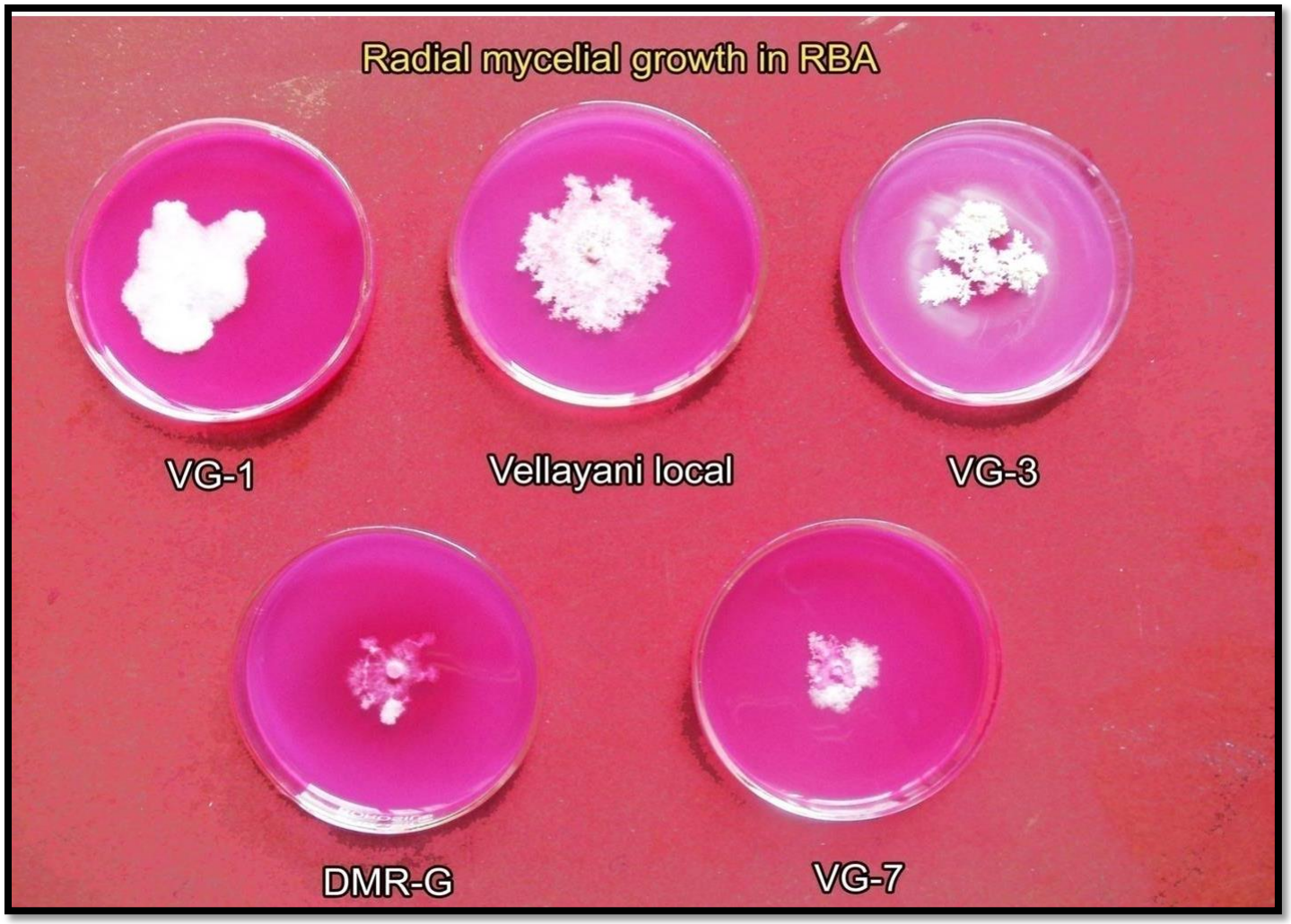


Plate 11. Mycelial growth of 5 isolates in Rose Bengal Agar medium

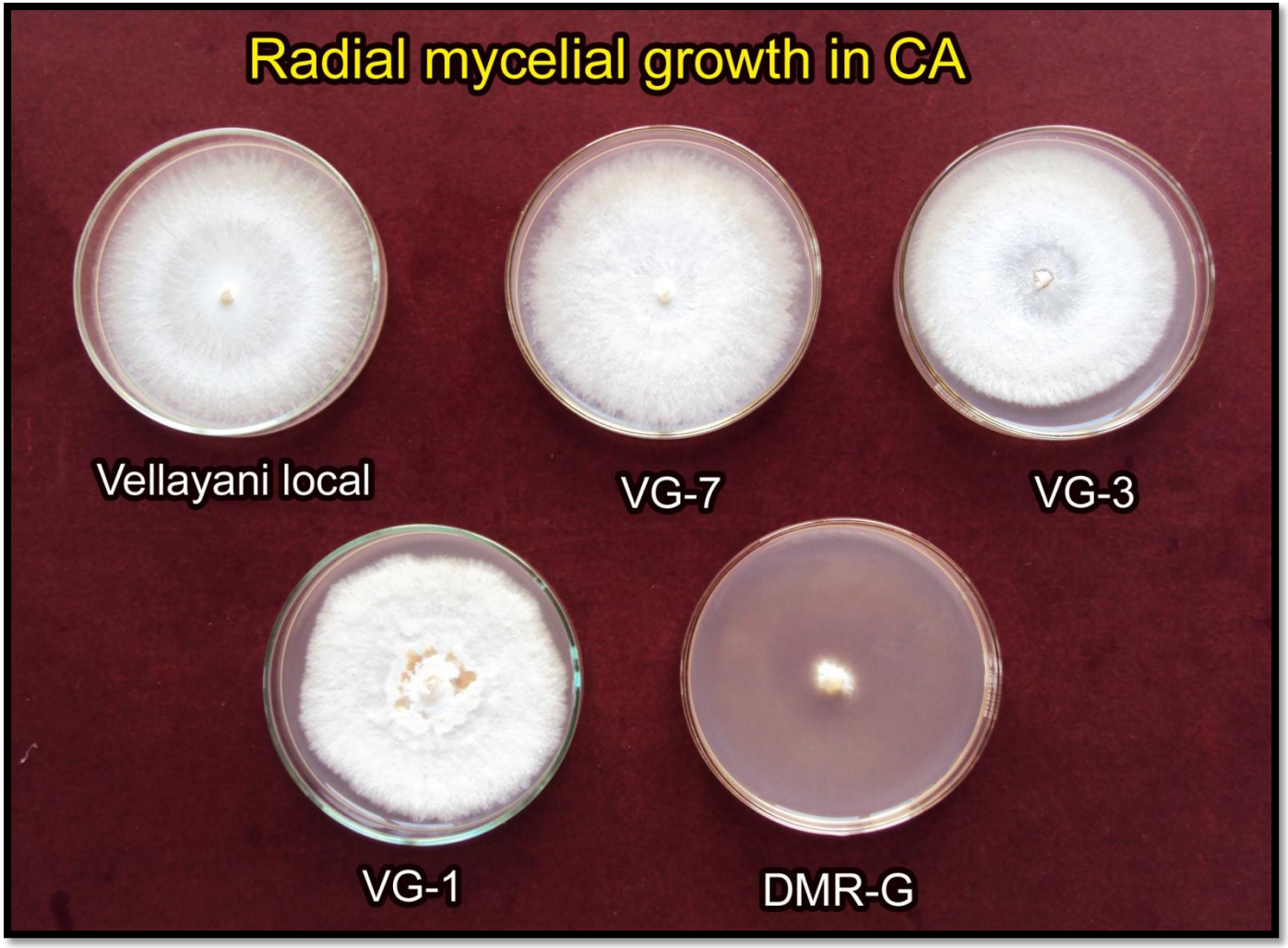


Plate 12. Mycelial growth of 5 isolates in Carrot Agar medium

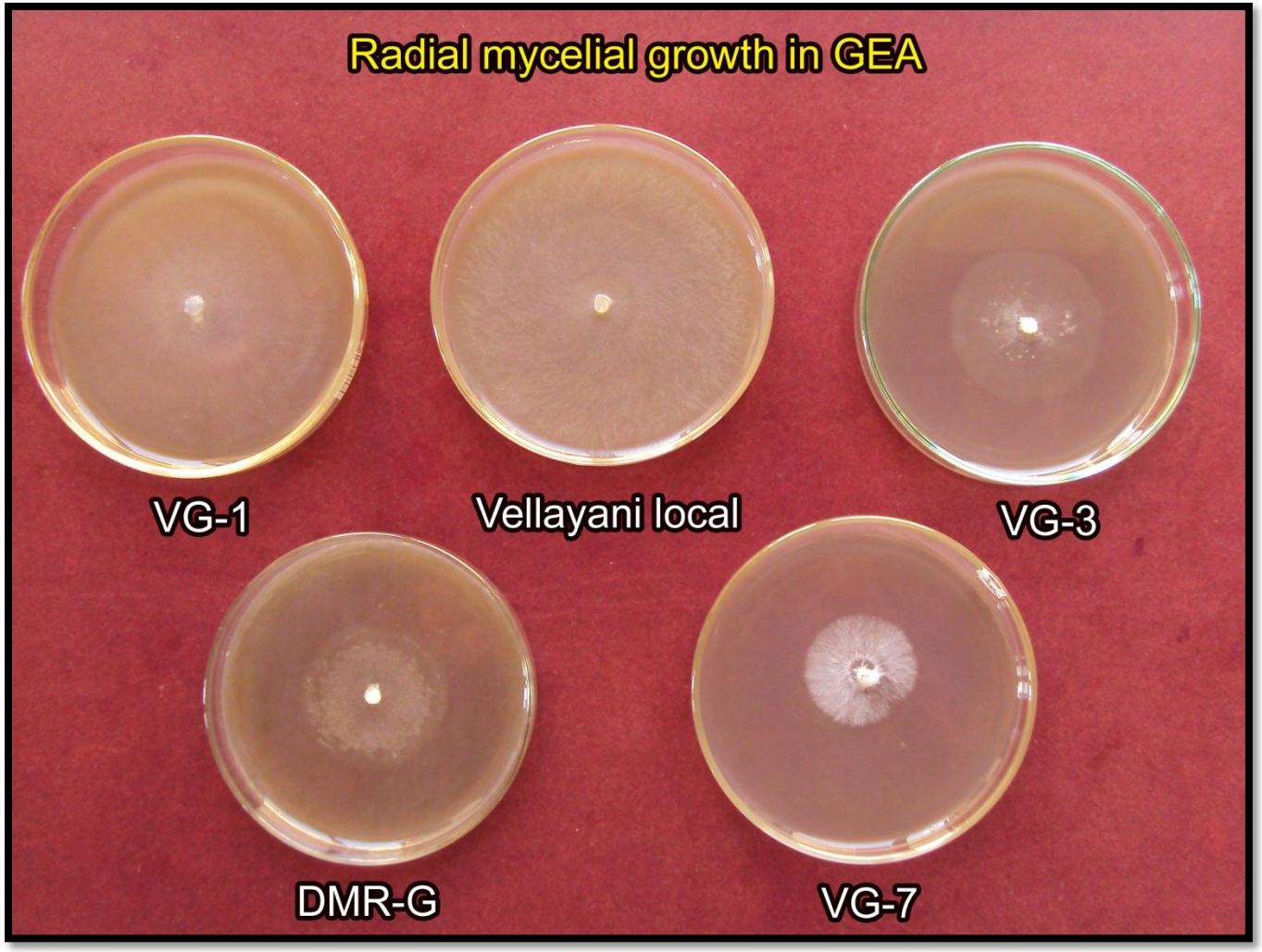


Plate 13. Mycelial growth of 5 isolates in *Ganoderma* Extract Agar medium

	RADIAL MYCELIAL GROWTH (cm)*					
ISOLATE	**PDA**	**OMA**	**RBA**	**CA**	**GEA**	**MEAN**
VG-1	7.02	5.46	0.64	8.60	2.86	5.00
VG-3	4.26	5.16	0.94	7.86	4.26	4.50
VG-7	6.12	7.32	1.40	8.32	8.68	6.37
Vellayani local	9.00	9.00	1.30	9.00	8.82	7.42
DMR-G	2.38	5.82	0.74	7.46	3.90	4.06
MEAN	5.76	6.56	1.00	8.23	5.70	
CD (at 5% levels) Cultures : 0.163 Treatments : 0.163 Interaction : 0.364						

** Average of 5 replications*

Table 4. Radial mycelial growth (cm) of *Ganoderma* isolates in different solid media (1 week growth)

Growth of *Ganoderma* isolates in different pH level (cm) *				
	pH 6	**pH 7**	**pH 8**	**Mean (Cultures)**
Vellayani local	8.76	8.90	7.83	8.50
VG-7	7.58	6.97	7.33	7.29
Mean (Treatments)	8.17	7.94	7.58	
CD (at 5% levels) Cultures : 0.035 Treatments : 0.043 Interaction : 0.061				

** Average of 6 replications*

Table 5. Growth of *Ganoderma* isolates in different pH levels (cm)

that of VG-7. Vellayani local isolate showed least growth in pH 8 while VG-7 grew the least in pH 7 (Table 5).

4.5.3. Effect of temperature on the mycelial growth of *Ganoderma*

Three different temperature conditions of 4ºC, ambient temperature and 35ºC were evaluated for the identification of best temperature suitable for the culture growth of *Ganoderma*. The result showed that different temperature conditions differ significantly in influencing radial growth (Plates 14, 15). Vellayani local isolate was found to grow well under ambient temperature and 35ºC. At 4ºC, no mycelial growth was recorded. While Vellayani local grew best under 35ºC, VG-7 showed maximum growth under ambient conditions (around 30 ºC) (Table 6).

4.5.4. Effect of light conditions on the mycelial growth of *Ganoderma*

Three different light conditions of artificial light, normal light and darkness were tested for the efficacy in the production of maximum radial mycelial growth. The result showed that different light conditions have no significant effect in influencing radial growth (Plate 16) (Table 7).

4.6. SPAWN PRODUCTION

Eight different substrates including four grain and four sawdust based substrates were evaluated for their efficacy as spawning substrate. The best spawn substrate was selected based on the minimum time taken for the spawn run and the nature of growth (Table 8).

Wheat was found to be the best substrate followed by sorghum and paddy. Both the isolates Vellayani local and VG-7 grew as thick white, fluffy mycelium all over the wheat grains within 13.50 days. The growth was thick and the spawn was

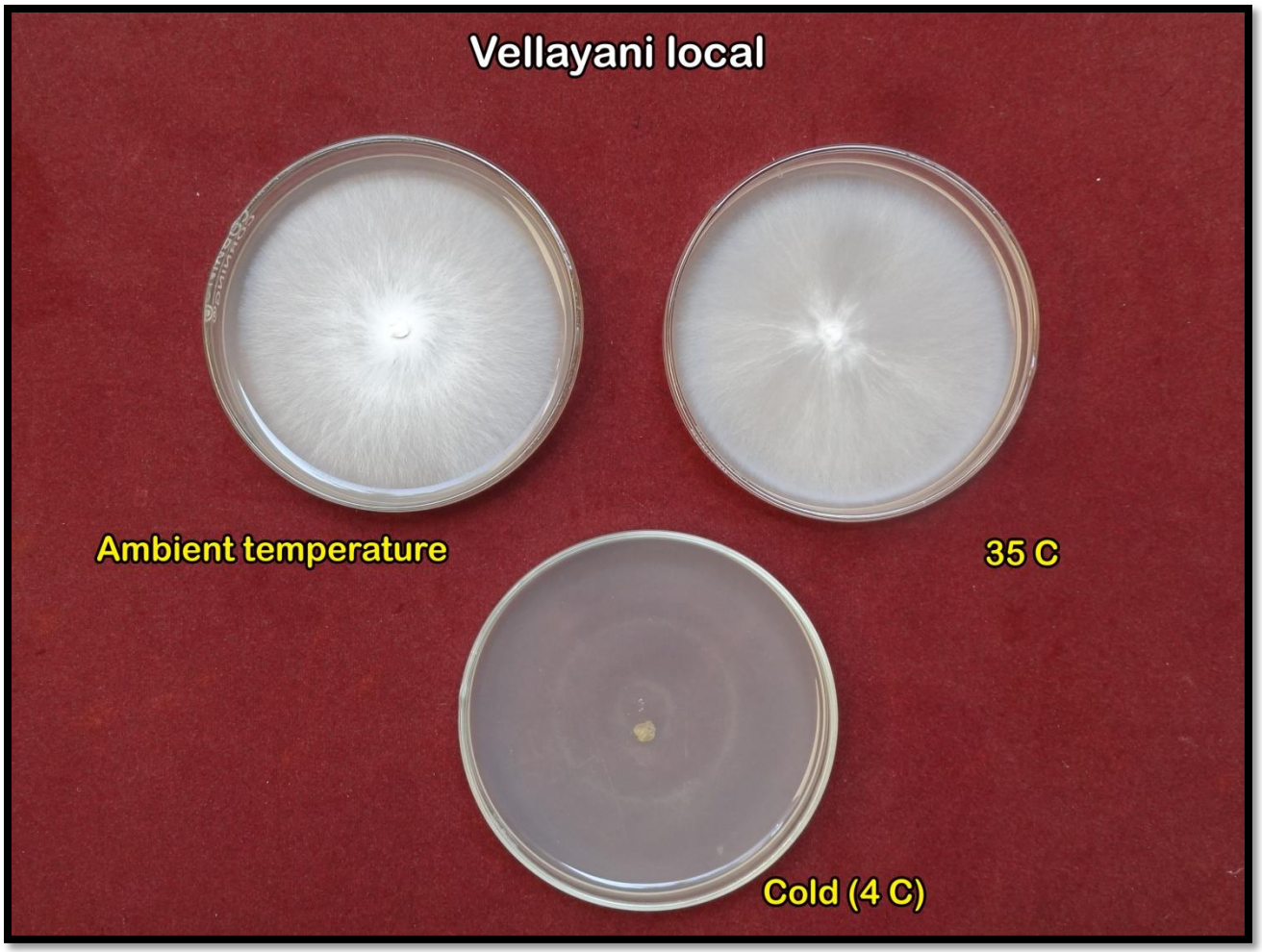


Plate 14. Comparison of *in vitro* growth of Vellayani local in various temperature conditions

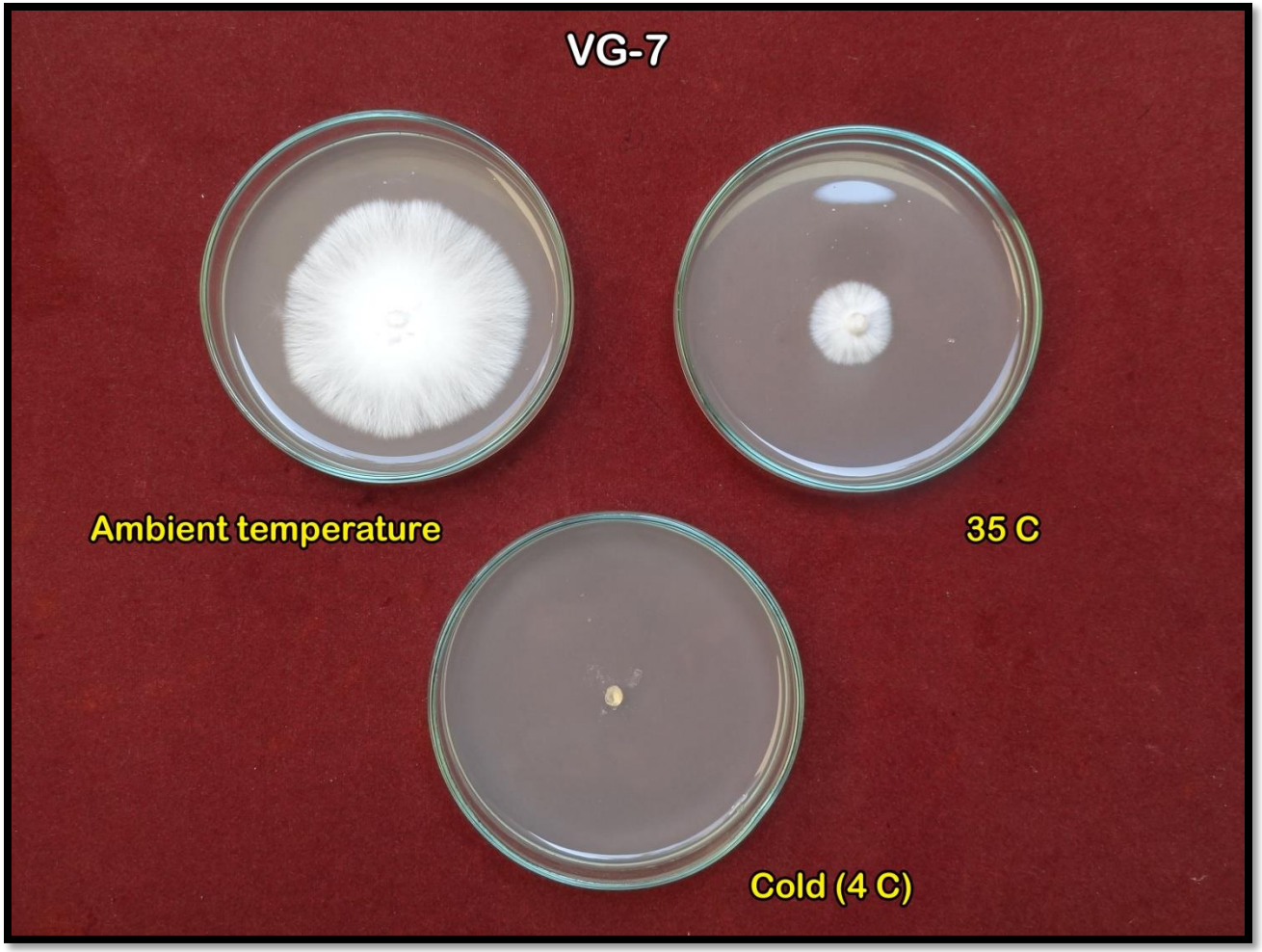


Plate 15. Comparison of *in vitro* growth of VG-7 in various temperature conditions

Growth of *Ganoderma* isolates in different temperatures (cm) *				
	Ambient temperature	**35^{0}c**	**Cold (4^{0}C)**	**Mean (Cultures)**
Vellayani local	8.24	8.70	0	8.47
VG-7	6.42	2.37	0	4.40
Mean (Treatments)	7.33	5.54	0	
CD (at 5% levels) Cultures : 0.057 Treatments : 0.024 Interaction : 0.034				

**Average of 6 replications*

Tabe 6. Growth of *Ganoderma* isolates in different temperatures (cm)

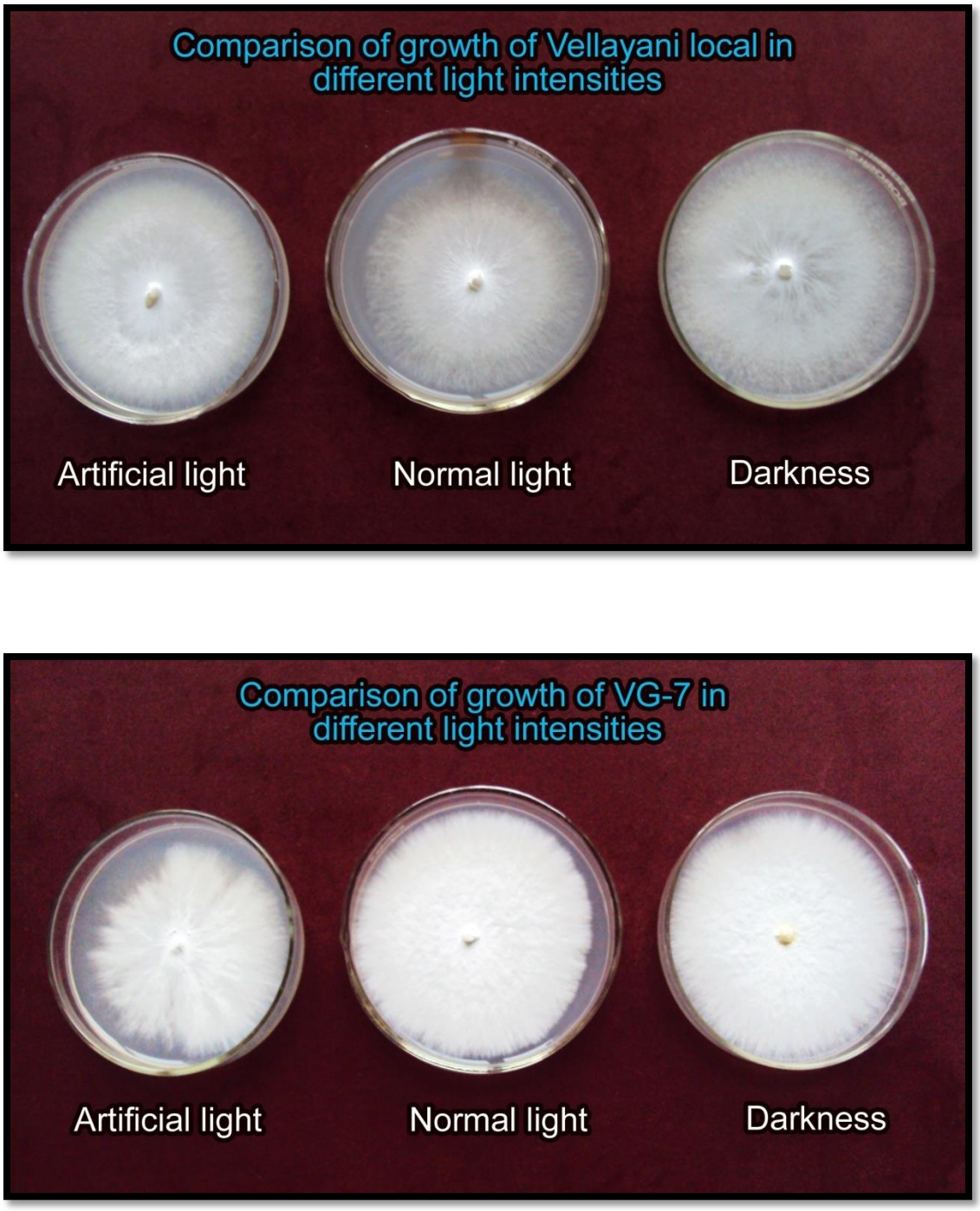


Plate 16. Comparison of *in vitro* growth of *Ganoderma* cultures in different light intensities

Growth of *Ganoderma* isolates in different light conditions (cm) *				
	Artificial light	**Normal light**	**Darkness**	**Mean (Cultures)**
Vellayani local	9.0	8.23	8.52	8.58
VG-7	7.69	8.32	8.30	8.10
Mean (Treatments)	8.35	8.28	8.41	
CD (at 5% levels) Cultures : 0.046 Treatments : NS Interaction : 0.080				

**Average of 6 replications*

Table 7. Growth of *Ganoderma* isolates in different light conditions (cm)

Plate 17 a. Growth of *Ganoderma* cultures in Paddy grain spawn

Plate 17 b. Growth of *Ganoderma* cultures in Wheat grain spawn

Plate 18 a. Growth of *Ganoderma* cultures in Sorghum grain spawn

Plate 18 b. Growth of *Ganoderma* cultures in Horsegram grain spawn

Plate 19 a. Growth of *Ganoderma* cultures in sawdust + rice bran spawn

Plate 19 b. Growth of *Ganoderma* cultures in sawdust + wheat bran spawn

Plate 20. Sporocarp arising from 2 months old mother spawn of Vellayani local

highly compact and with ageing, spawn showed slight yellowish colouration in both the isolates (Plate 17 b).

Sorghum grains were colonized by the *Ganoderma* isolates in 14.79 days. While Vellayani local spread as thick white mycelial growth all over the grain making it highly compact, VG-7 colonised the grains in an irregular and non uniform manner as patches. The spawn was loose and slight yellowish pigmentation was observed (Plate 18 b).

Paddy grains were fully colonized by *Ganoderma* cultures in 19.42 days. The growth of Vellayani local in paddy grains was thicker and fluffier than the VG-7 isolate and the spawn was compact in the former case while it was loose in VG-7 inoculated packets. Though wheat and sorghum grains were better, paddy spawn was further used for cultivation of *Ganoderma* owing to its cost effectiveness and easy availability (Plate 17 a).

Horse gram was found to be a suitable substrate for Vellayani local isolate in terms of time required for colonization but the mycelial density was very sparse. While Vellayani local isolate grew as uniform thin mycelium around the grains, VG-7 isolate colonized them as thin cobweb like mycelium. Thick white areas were seen only around the point of inoculation. 21.14 days were taken to complete the mycelial run in horse gram (Plate 18 a).

Among the different sawdust based substrates tested, only sawdust and wheat bran combination provided favourable conditions for spawn growth. Also, rate of contamination was high when sawdust substrates were used. These materials took twenty one to thirty three days for complete mycelial colonization (Plate 19 a, b). Sporocarp formation from the mother spawn was observed in Vellayani local after two months (Plate 20).

Time taken for full colonisation of spawn substrates by cultures VG-7 and Vellayani local (days)									
Treatments	**Paddy**	**Wheat**	**Horse Gram**	**Sorghum**	**HW SD**	**SW SD**	**SD + RB**	**SD + WB**	**Mean (cultures)**
VG-7	19.85	14.42	28.14	15.57	30.00	32.57	28.85	27.42	24.60
Vellayani local	18.99	12.57	14.14	14.0	27.57	27.57	25.57	21.42	20.23
Mean (substrates)	19.42	13.50	21.14	14.79	28.79	30.07	27.21	24.42	

CD (at 5% levels):- Cultures: 0.026; Substrates: 0.052; Interaction: 0.074

HW SD: Hardwood sawdust; SW SD: Softwood sawdust;

SD + RB: Sawdust + rice bran; SD + WB: Sawdust + wheat bran

Table 8. Time taken for full colonisation of spawn substrates by cultures VG-7 and Vellayani local (days)

4.7. CULTIVATION

The experimental trials were conducted to evaluate the efficacy of eight bed substrates *viz.* sawdust (90%) + rice bran (10%), sawdust (90%) + wheat bran (10%), wood chips (80%) + rice bran(20%), wood chips (80%) + wheat bran (20%), sawdust (80%) + rice bran (20%), sawdust (80%) + wheat bran (20%), sawdust (80%) + rice bran (18%) + CaCO3 (1%) + sucrose (1%) and sawdust (78%) + rice bran (20%) + $CaCO_3$ (2%) for the production of *Ganoderma* sporocarps. Two different isolates, Vellayani local and VG-7 were grown in these substrates (Plate 21, 22).

4.7.1. Time taken for complete mycelial colonization of the bed substrates

Among the two isolates grown in bed substrates, Vellayani local took significantly shorter time for the complete mycelial colonization in four substrate combinations consisting of sawdust and bran alone i.e.; sawdust (90%) + rice bran (10%), sawdust (90%) + wheat bran (10%), sawdust (80%) + rice bran (20%), sawdust (80%) + wheat bran (20%). There was significant difference between cultures and treatments in the time required for mycelial colonization in the substrates. The isolate Vellayani local took the shortest time of 18.33 days to entirely colonise the substrate sawdust (90%) + wheat bran (10%) which was on par with the substrate sawdust (90%) + rice bran (10%) in which it took 18.50 days. However, VG-7 performed the best in sawdust (90%) + rice bran (10%) which took 20.17 days to colonise the substrate completely and was significantly different from the other substrates used. So, it can be concluded that sawdust (90%) + rice bran (10%) is the best among the eight substrates evaluated for mycelial colonization of the fungus *Ganoderma* (Table 9).

4.7.2. Time taken for primordial initiation

Among the two isolates evaluated, Vellayani local took significantly shorter time for the primordial initiation in all the substrates. The two substrates containing

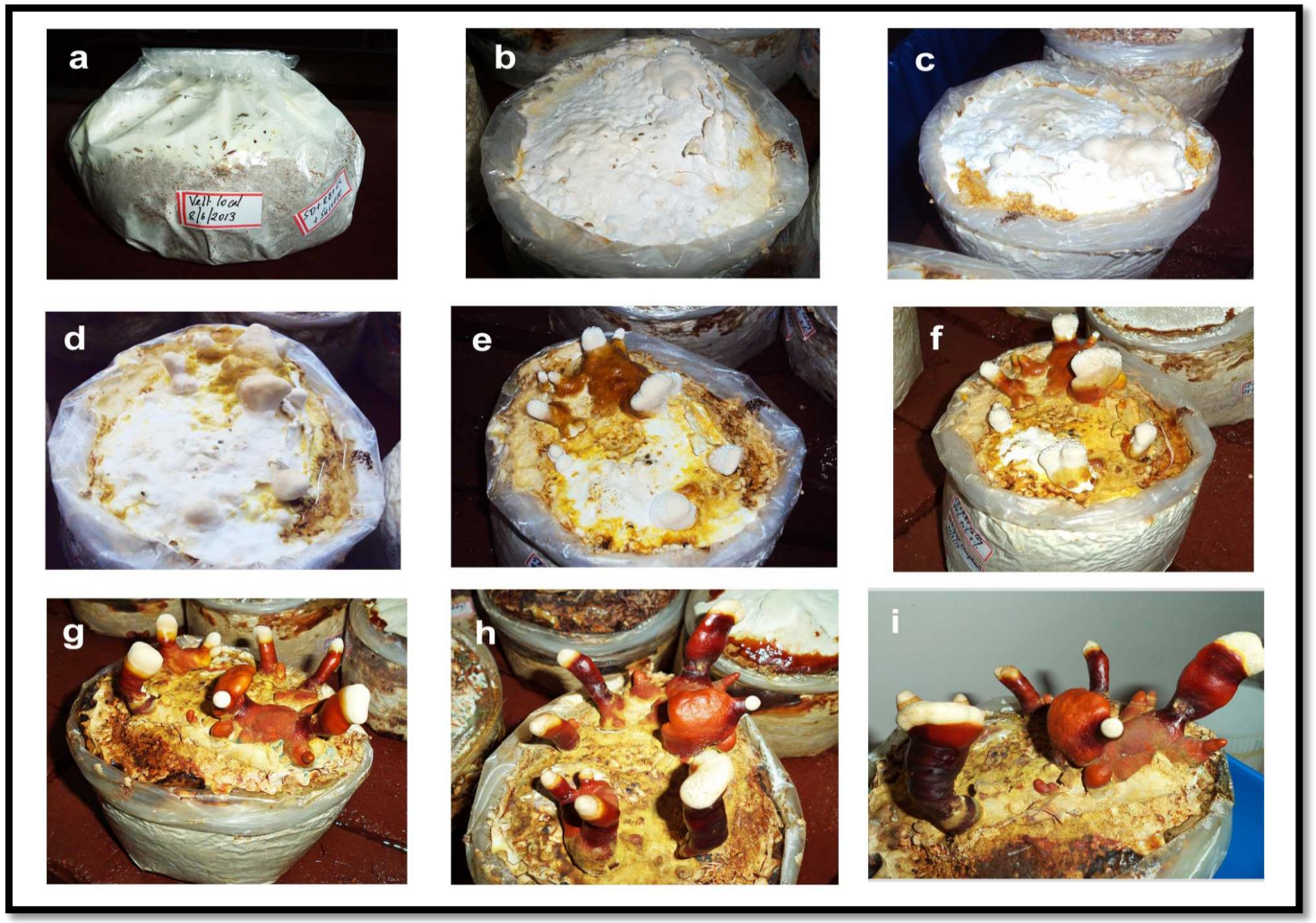


Plate 21. Growth stages of Vellayani local

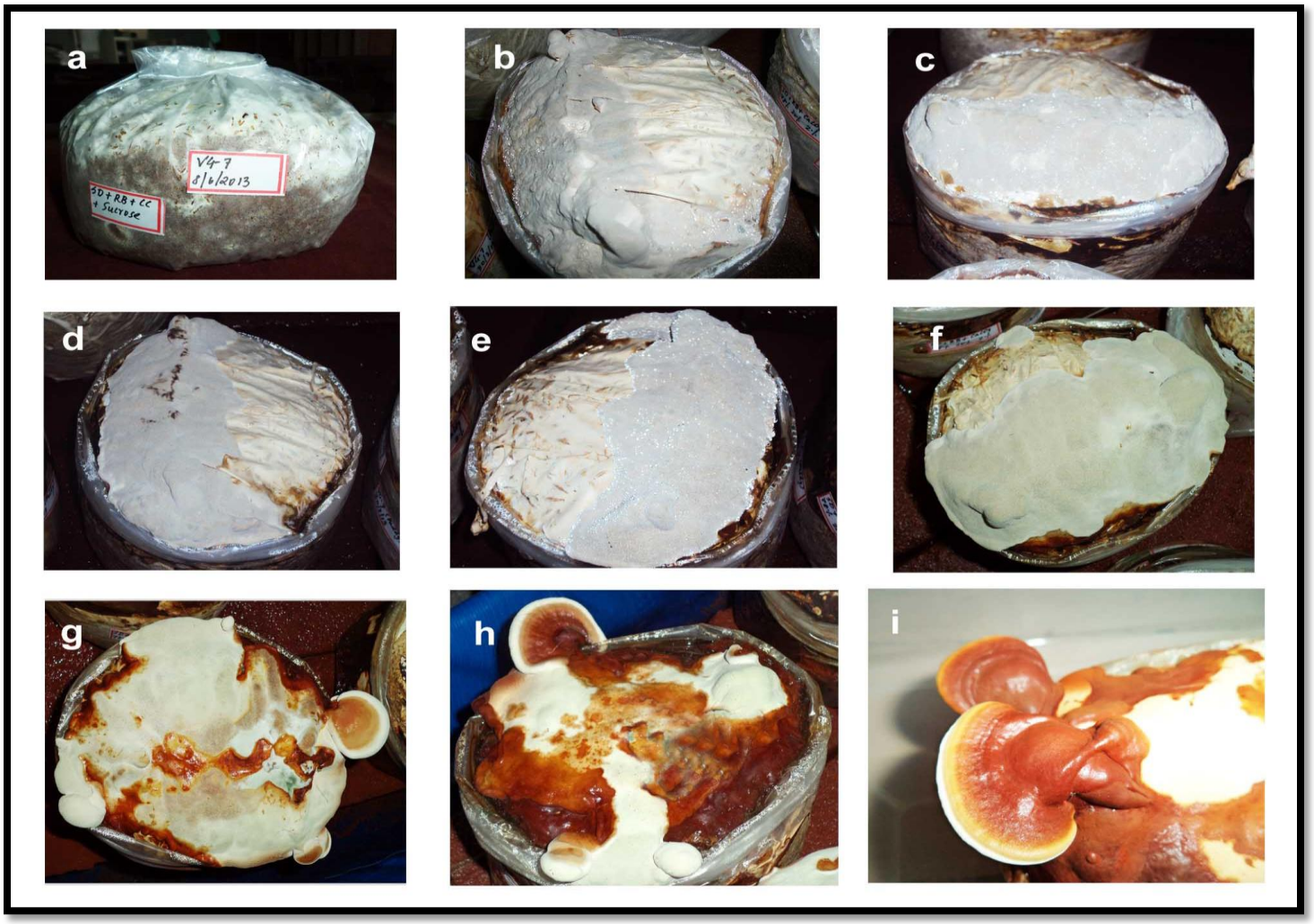


Plate 22. Growth stages of VG-7

Time taken for complete mycelial colonization of mushroom beds in different substrates (days) *									
Treatments	**SD (90%) + RB (10%)**	**SD (90%) + WB (10%)**	**Wood chips (80%) + RB (20%)**	**Wood chips (80%) + WB (20%)**	**SD (80%) + RB (20%)**	**SD (80%) + WB (20%)**	**SD (80%) + RB (18%) + $CaCO_3$ (1%) + Sucrose (1%)**	**SD (78%) + RB (20%) + $CaCO_3$ (2%)**	**Mean (Cultures)**
Vellayani local	18.50	18.33	29.00	29.17	21.83	20.67	25.33	21.33	23.02
VG-7	20.17	22.33	28.00	28.33	22.50	21.00	25.00	21.17	23.56
Mean (Treatments)	19.33	20.33	28.50	28.75	22.17	20.83	25.17	21.25	

* *Average of 6 replications*

CD values (at 5% levels): Substrates: 0.707288; Cultures: 0.353644; Su x Cu: 1.000256

Table 9. Time taken for complete mycelial colonization of mushroom beds in different substrates (days)

wood chips did not develop any pinheads even after incubation for a month. It took the shortest time of 12.33 days for primordial formation by the isolate Vellayani local in the substrates sawdust (80%) + wheat bran (20%) and sawdust (78%) + rice bran (20%) + $CaCO_3$ (2%) which was statistically on par with 12.83 days taken in sawdust (80%) + rice bran (20%) and sawdust (90%) + wheat bran (10%). The primordial formation by VG-7 was the earliest at 20.67 days in sawdust (80%) + wheat bran (20%) which was statistically on par with the 21.50 days taken in sawdust (78%) + rice bran (20%) + $CaCO_3$ (2%) and sawdust (90%) + rice bran (10%). Hence, sawdust (80%) + wheat bran (20%) was found to be the most suitable substrate for primordial initiation in *Ganoderma* (Table 10).

4.7.3. Time taken for sporophore formation

There was no significant difference between the cultures regarding the time taken for fruiting body production. Between the substrates, sawdust (78%) + rice bran (20%) + $CaCO_3$ (2%) was ideal for early fruiting of both strains mushrooms, in 20.33 days, followed by sawdust (90%) + wheat bran (10%) which enabled fruiting in below 20.92 days (Table 11). The wood chips was found unsuitable for sporophore production with rice bran as well as wheat bran.

4.7.4. Total crop growth period

Among the cultures, total crop growth period was least for Vellayani local. Vellayani local inoculated substrate sawdust (90%) + wheat bran (10%) completed its growth and developed mature sporocarps within 51.83 days which was significantly different from other substrates. The culture VG-7 completed its growth first in the substrates sawdust (80%) + wheat bran (20%) and sawdust (78%) + rice bran (20%) + $CaCO_3$ (2%) both in 63.17 days. This was followed by completion of its growth cycle in 63.33 days in the substrate sawdust (90%) + rice bran (10%) which was on par with the former (Table 12).

Time taken for primordial formation in mushroom beds of different substrates (days) *									
Treatments	**SD (90%) + RB (10%)**	**SD (90%) + WB (10%)**	**Wood chips (80%) + RB (20%)**	**Wood chips (80%) + WB (20%)**	**SD (80%) + RB (20%)**	**SD (80%) + WB (20%)**	**SD (80%) + RB (18%) + $CaCO_3$ (1%) + Sucrose (1%)**	**SD (78%) + RB (20%) + $CaCO_3$ (2%)**	**Mean (Cultures)**
Vellayani local	13.83	12.83	No growth	No growth	12.83	12.33	15.33	12.33	13.25
VG-7	21.50	21.83	No growth	No growth	22.17	20.67	24.50	21.50	22.03
Mean (Treatments)	17.67	17.33	No growth	No growth	17.50	16.50	19.92	16.92	

* *Average of 6 replications*

CD values (at 5% levels): Substrates: 0.9660918; Cultures: 0.5577733; Su x Cu: 1.36626

Table 10. Time taken for primordial formation in mushroom beds of different substrates (days)

Time taken for sporophore formation in mushroom beds of different substrates (days) *									
Treatments	**SD (90%) + RB (10%)**	**SD (90%) + WB (10%)**	**Wood chips (80%) + RB (20%)**	**Wood chips (80%) + WB (20%)**	**SD (80%) + RB (20%)**	**SD (80%) + WB (20%)**	**SD (80%) + RB (18%) + $CaCO_3$ (1%) + Sucrose (1%)**	**SD (78%) + RB (20%) + $CaCO_3$ (2%)**	**Mean (Cultures)**
Vellayani local	21.17	20.67	No growth	No growth	23.50	21.83	22.17	20.17	21.58
VG-7	21.67	21.17	No growth	No growth	22.50	21.50	22.00	20.50	21.56
Mean (Treatments)	21.42	20.92	No growth	No growth	23.00	21.67	22.08	20.33	

* *Average of 6 replications*

CD values (at 5% levels): Substrates: 0.8745286; Cultures: 0.5049094; Su x Cu: 1.23677

Table 11. Time taken for sporophore formation in mushroom beds of different substrates (days)

Total crop growth period (days) *									
Treatments	**SD (90%) + RB (10%)**	**SD (90%) + WB (10%)**	**Wood chips (80%) + RB (20%)**	**Wood chips (80%) + WB (20%)**	**SD (80%) + RB (20%)**	**SD (80%) + WB (20%)**	**SD (80%) + RB (18%) + $CaCO_3$ (1%) + Sucrose (1%)**	**SD (78%) + RB (20%) + $CaCO_3$ (2%)**	**Mean (Cultures)**
Vellayani local	53.50	51.83	Incomplete	Incomplete	58.17	54.83	62.83	53.83	55.83
VG-7	63.33	65.33	Incomplete	Incomplete	67.17	63.17	71.50	63.17	65.61
Mean (Treatments)	58.42	58.58	Incomplete	Incomplete	62.67	59.00	67.17	58.50	

** Average of 6 replications*

CD values (at 5% levels): Substrates: 1.627349; Cultures: 0.9395502; Su x Cu: 2.301419

Table 12. Total crop growth period in different substrates (days)

4.7.5. Total number of fruiting bodies

Among the cultures, Vellayani local produced more sporocarps in beds than VG-7 in all substrates except sawdust (80%) + rice bran (18%) + CaCO3 (1%) + sucrose (1%) in which VG-7 produced more number of fruiting bodies (2.83) which was significantly on par with Vellayani local (2.33). Among the substrates, maximum fruiting bodies (5.92) were produced in sawdust (80%) + wheat bran (20%).

4.7.6. Nature and colour of sporocarps

There was wide variation in the morphology of Vellayani local and VG-7 fruiting bodies but there were no much differences observed due to difference in substrates.

The sporocarps from Vellayani local beds were deep reddish brown in colour, brilliantly laccate, stipitate and had a polished or glossy appearance. The pileus was whitish, with slight orange tinge and may or may not be well differentiated from the stipe. The morphology of the sporocarps varied tremendously from bed to bed. However, most of the fruiting bodies were slender, highly branched and shiny.

The fruiting bodies from VG-7 beds were brownish, of woody consistency, either laccate or non laccate, sessile and non polished. The pileus was brown with a white margin. All the sporocarps were bracket shaped and thick. The number of sporocarps per bed was less compared to Vellayani local.

4.7.7. Total biological yield

The biological yield in gram per mushroom bed was recorded by weighing the whole fruiting bodies harvested from each bed. Among the two isolates, VG-7 recorded maximum yield in all the substrates evaluated. The highest yield of VG-7 was 27.41 g which was obtained in the substrate sawdust (80%) + rice bran (20%) followed by 25.32 g in sawdust (80%) + wheat bran (20%). These two results were

Total number of fruiting bodies *									
Treatments	**SD (90%) + RB (10%)**	**SD (90%) + WB (10%)**	**Wood chips (80%) + RB (20%)**	**Wood chips (80%) + WB (20%)**	**SD (80%) + RB (20%)**	**SD (80%) + WB (20%)**	**SD (80%) + RB (18%) + $CaCO_3$ (1%) + Sucrose (1%)**	**SD (78%) + RB (20%) + $CaCO_3$ (2%)**	**Mean (Cultures)**
Vellayani local	7.00	8.17	Nil	Nil	4.50	9.17	2.33	6.17	6.22
VG-7	3.33	2.67	Nil	Nil	3.83	2.67	2.83	1.83	2.86
Mean (Treatments)	5.17	5.42	-	-	4.17	5.92	2.58	4.00	

* *Average of 6 replications*

CD values (at 5% levels): Substrates: 1.584064 ;Cultures: 0.9145598; Su x Cu: 2.240205

Table 13. Total number of fruiting bodies

Total biological yield (g) *									
Treatments	**SD (90%) + RB (10%)**	**SD (90%) + WB (10%)**	**Wood chips (80%) + RB (20%)**	**Wood chips (80%) + WB (20%)**	**SD (80%) + RB (20%)**	**SD (80%) + WB (20%)**	**SD (80%) + RB (18%) + $CaCO_3$ (1%) + Sucrose (1%)**	**SD (78%) + RB (20%) + $CaCO_3$ (2%)**	**Mean (Cultures)**
Vellayani local	15.40	15.52	Incomplete	Incomplete	15.45	18.34	13.85	15.76	15.72
VG-7	25.04	22.64	Incomplete	Incomplete	27.41	25.32	23.08	20.59	24.01
Mean (Treatments)	20.22	19.08	Incomplete	Incomplete	21.43	21.83	18.46	18.18	

* *Average of 6 replications*

CD values (at 5% levels): Substrates: 2.266294; Cultures: 1.308446; Su x Cu: 3.205024

Table 14. Total biological yield from different substrates (g)

on par with each other. For the isolate Vellayani local, the highest yield recorded was 18.34 grams in the substrate sawdust (80%) + wheat bran (20%) which was significantly different from the next highest yield of 15.76 obtained in sawdust (78%) + rice bran (20%) + $CaCO_3$ (2%) (Table 14).

4.7.8. Biological Efficiency (BE)

Among the two isolates, VG-7 recorded maximum biological efficiency in all the substrates evaluated. The highest biological efficiency of VG-7 was 6.59% which was obtained in the substrate sawdust (80%) + rice bran (20%) followed by 6.09% in sawdust (80%) + wheat bran (20%). These two results were on par with each other upon statistical analysis. For the isolate Vellayani local, highest biological efficiency recorded was 4.41% in the substrate sawdust (80%) + wheat bran (20%) which was significantly different from the next highest biological efficiency of 3.79% obtained in sawdust (78%) + rice bran (20%) + $CaCO_3$ (2%) (Table 15).

4.8. PROXIMATE CONSTITUENT ANALYSIS

The proximate constituents of both the isolates Vellayani local and VG-7 were analysed and the results are presented (Table 16).

The proximate constituents of two isolates of *Ganoderma* mushroom were evaluated using standard technique as described in review. The moisture content of *Ganoderma* was found to be 58.2% and 53.4% in Vellayani local and VG-7 respectively (fresh mushroom). Protein content present in the mushroom was estimated using Kjeldahl's method and it was found to be 20.32% and 21.75% in Vellayani local and VG-7 respectively. Fat content present in the mushroom was estimated using soxhlet extraction method and it was found to be 4.1% and 2.0% in Vellayani local and VG-7 respectively. Carbohydrate content present in *Ganoderma* mushroom was estimated by anthrone method and it was found to be 18.25% in

Biological Efficiency (%) *									
Treatments	**SD (90%) + RB (10%)**	**SD (90%) + WB (10%)**	**Wood chips (80%) + RB (20%)**	**Wood chips (80%) + WB (20%)**	**SD (80%) + RB (20%)**	**SD (80%) + WB (20%)**	**SD (80%) + RB (18%) + $CaCO_3$ (1%) + Sucrose (1%)**	**SD (78%) + RB (20%) + $CaCO_3$ (2%)**	**Mean (Cultures)**
Vellayani local	3.70	3.73	Incomplete	Incomplete	3.72	4.41	3.33	3.79	3.78
VG-7	6.02	5.44	Incomplete	Incomplete	6.59	6.09	5.55	4.95	5.77
Mean (Treatments)	4.86	4.59	Incomplete	Incomplete	5.15	5.25	4.44	4.37	

* *Average of 6 replications*

CD values (at 5% levels): Substrates: 0.5447031; Cultures: 0.3144845; Su x Cu: 0.7703265

Table 15. Biological efficiency in different substrates (%)

Vellayani local and 20.50 in VG-7. The ash content present in the isolates of the mushroom was found to be 1% and 1.23% for Vellayani local and VG-7 respectively. Fibre content present in *Ganoderma* was estimated and it was found to be 9.87% in Vellayani local and 10.76% in VG-7.

4.9. SUBMERGED CULTURE PRODUCTION OF MUSHROOMS

The yield of two strains namely Vellayani local and VG-7 pellets produced by submerged cultivation in carrot broth and oatmeal broth was assessed by preweighing a sterile filter paper and filtering the contents through it and weighing it again. The difference in weight gave the biomass in grams.

It was observed that a yield of 9.86 g and 10.01 g was obtained for the culture Vellayani local in carrot broth and oatmeal broth respectively while VG-7 yielded 11.83 g and 12.35 g respectively in carrot broth and oatmeal broth.

Proximate constituent analysis of the two isolates of *Ganoderma* (%)			
Sl. No.	**Proximate constituent**	**Vellayani local**	**VG-7**
1	Moisture *	58.2	53.4
2	Protein#	20.32	21.75
3	Fat#	4.1	2.0
4	Carbohydrate#	18.25	20.50
5	Fibre#	9.87	10.76
6	Ash#	1.00	1.23

* - Presented in fresh weight basis

- Presented in dry weight basis

Table 16. Proximate constituent analysis of the two isolates of *Ganoderma* (%)

Discussion

5. DISCUSSION

During the course of this study, surveys were conducted in Kerala during 2011 and 2012 rainy seasons as a result of which 20 samples of *Ganoderma* sporocarps were collected and described. Moncalvo and Ryvarden (1998) have opined that *Ganoderma* is an organism that thrives very well in the hot humid conditions prevailing in the tropics and sub tropics. The details including location, date of collection, host tree, condition of the host, soil type, vegetation, morphology and age of the sporocarps were noted. Survey, collection and description of *Ganoderma* spp. has been attempted by different workers all over the world (Jha *et al.*, 2011; Sankaran *et al.*, 2005; Sharma *et al.*, 2012).

The trees from which the sporocarps of *Ganoderma* were collected during the survey in the present study include *Anacardium occidentale, Areca catechu, Artocarpus hirsutus, Cocos nucifera*, *Macaranga hypoleuca*, *Manilkara* spp. *and Peltophorum ferrugineum.* The sporocarps were observed on dried tree stumps and live trees also and they carried in their coloration from whitish, cream colored, brown colored to deep red coloration. Wasser (2005) has reported the frequent occurrence of *Ganoderma* species on many tree species including oak, maple, elm, willow, sweet gum magnolia etc. Sankaran and his co workers in 2008 have observed the incidence of many *Ganoderma* spp. causing diseases of perennial crops in India. They recorded 144 hosts in India which includes most of the tree hosts observed in the present study and the major pathogenic species reported were *G. lucidum* and *G. applanatum.*

During the present study five *Ganoderma lucidum* isolates were used of which three isolates of *Ganoderma* sporocarps were got during the survey were obtained in pure culture which were named as VG-1, VG-3 and VG-7 and apart from this, a culture, DMR-G, was obtained from Directorate of Mushroom research, Solan

and another culture, Vellayani local was procured from Instructional farm, Vellayani. Gottlieb and Wright (1999) while studying 124 specimens of *Ganoderma* from South America observed that correlation between morphological features and isoenzyme patterns could not be established in this group because of the high phenotypic plasticity. Sharma and co-workers in 2012 conducted survey, isolation and characterization of *Ganoderma* spp. from Chattisgarh and majority of the isolates obtained were that of *Ganoderma lucidum.* Jha *et al.*, (2011) surveyed the Nagarjun and Phulchowki regions of Kathmandu Valley in Nepal and collected three *Ganoderma* species including *G. applanatum, G. lucidum* and *G. tsugae*.

The chromatogram from the BLAST analysis of the sequences from different isolates showing the first 100 hits showed that, the isolates were found to have similarity with 15 sequences with more than 200 identity keys using ten available *Ganoderma* sequences. Sharma and co-workers in 2012 reported the molecular characterization of six isolates using RAPD and found that two isolates were found to differ from the rest four isolates as they fell into different clusters.

Mohanty *et al.* (2011) observed that there is high variability in basidiome morphology amongst *Ganoderma* spp. as observed in the present study and complicated speciation which often leads to inconclusive identification using traditional methods. They analysed the two isolates collected from *Acacia nilonica* and *Tectona grandis* using morphological characters and sequencing of ribosomal 5.8S RNA and the flanking ITS (Internal transcribed spacers) and confirmed the species as *G. resinaceum* and *G. weberianum*.

In the present study, the cultural characteristics of *Ganoderma* spp. including hyphal growth, pigmentation and chlamydospore production. Pigmentation was observed in three cultures after a week of growth due to the production of chlamydospores. The basidium and clamp connections were visible in the mycelia of VG-1 upon microscopic examination.

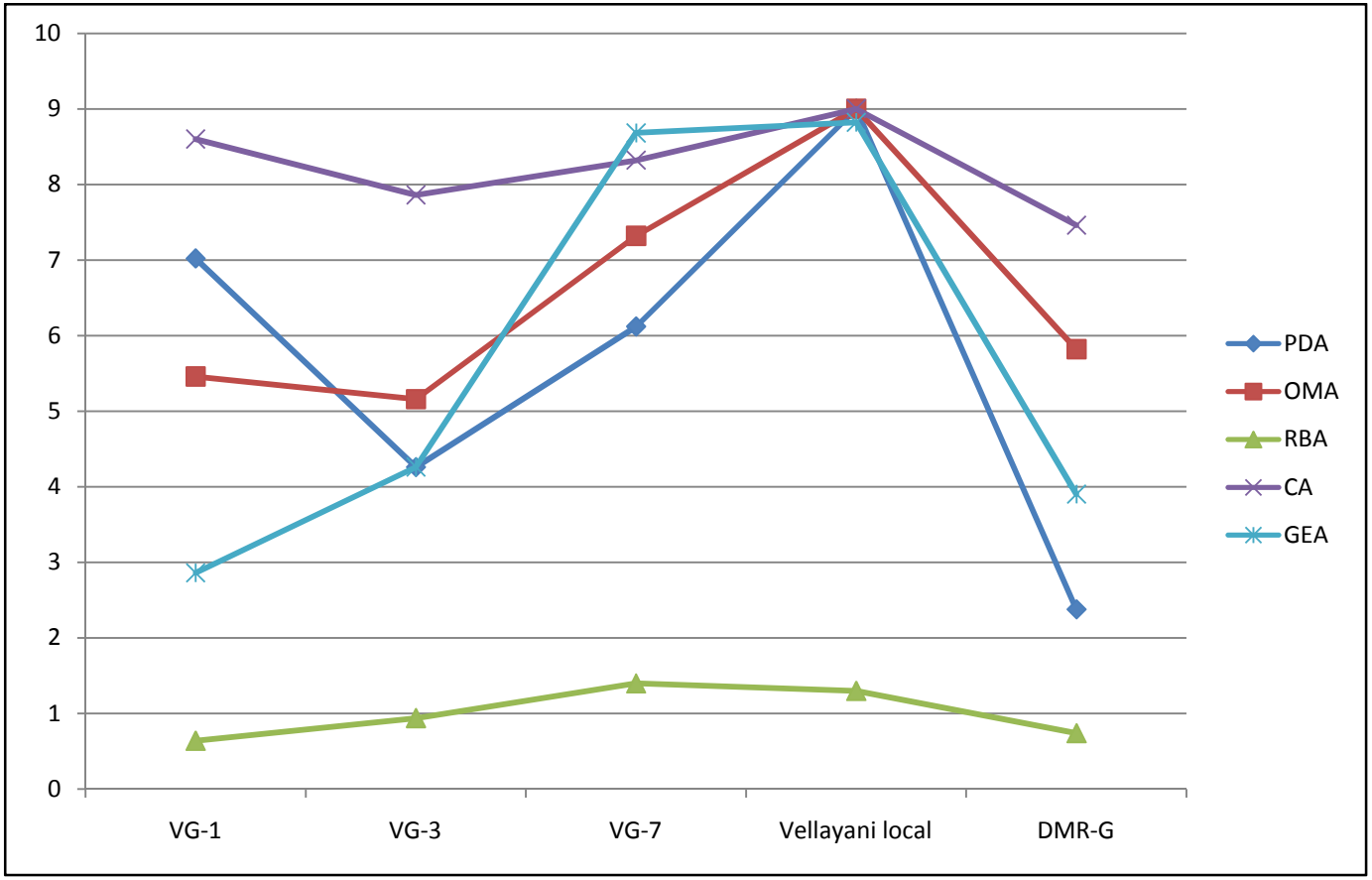


Fig. 1. Mycelial growth of five isolates of *Ganoderma* in different nutrient media (in cm)

Badalyan and associates in 2012 opined that *G. lucidum* produces numerous ovoid chlamydospores were formed by *Ganoderma* spp. as earlier suggested by Moncalvo *et.al.*, in 1995 which was observed in the present study also .

When five different nutrient media were evaluated for their efficacy in supporting the growth of five *Ganoderma* isolates *in vitro*, it was seen that among the cultures, Vellayani local was the fastest growing one and among the media used, carrot agar (CA) was found to be the best followed by oatmeal agar (OMA) and potato dextrose agar (PDA) (Fig. 1). Tseng *et al.*, in 1984 observed that synthetic media were poor for the maintenance of vigorous growth of *Ganoderma* when compared with PDA. Bilay *et al.* (2000) observed that the commercially available malt extract agar and potato dextrose agar to be the best media for growing *Ganoderma* spp. Zutshi and Gupta (2013) observed that malt extract agar was the best medium to support mycelial growth of *Ganoderma lucidum.*

The results of the present study indicated that slightly acidic pH of 6 was better suited for the growth of VG-7 isolate while Vellayani local preferred a neutral pH of 7 by producing a radial growth of 8.90 cm which was on par with pH 6 (8.76 cm) (Fig. 2). Jo *et al.*, in 2009 found that a pH range of 6-9 was most suited for supporting the growth of *G. applanatum.* The optimum pH range for the growth of *Ganoderma* spp. has been reported to be 5-9 (Cho *et al.*, 1993; Jayasinghe *et al.*, 2008) which is in corroboration with the results of this study.

The results of the present study showed that different temperature conditions differ significantly in influencing radial growth and the Vellayani local isolate was found to grow well under ambient temperature and 35ºC while at the cold range i.e., at 4ºC, no mycelial growth was recorded (Fig. 3). The culture VG-7 showed maximum growth under ambient conditions (around 30 ºC). Jo *et al.*, in 2009 found that a temperature range between 25-30 ºC was most suited for supporting the mycelial growth of *G. applanatum* and there was growth suppression at temperatures

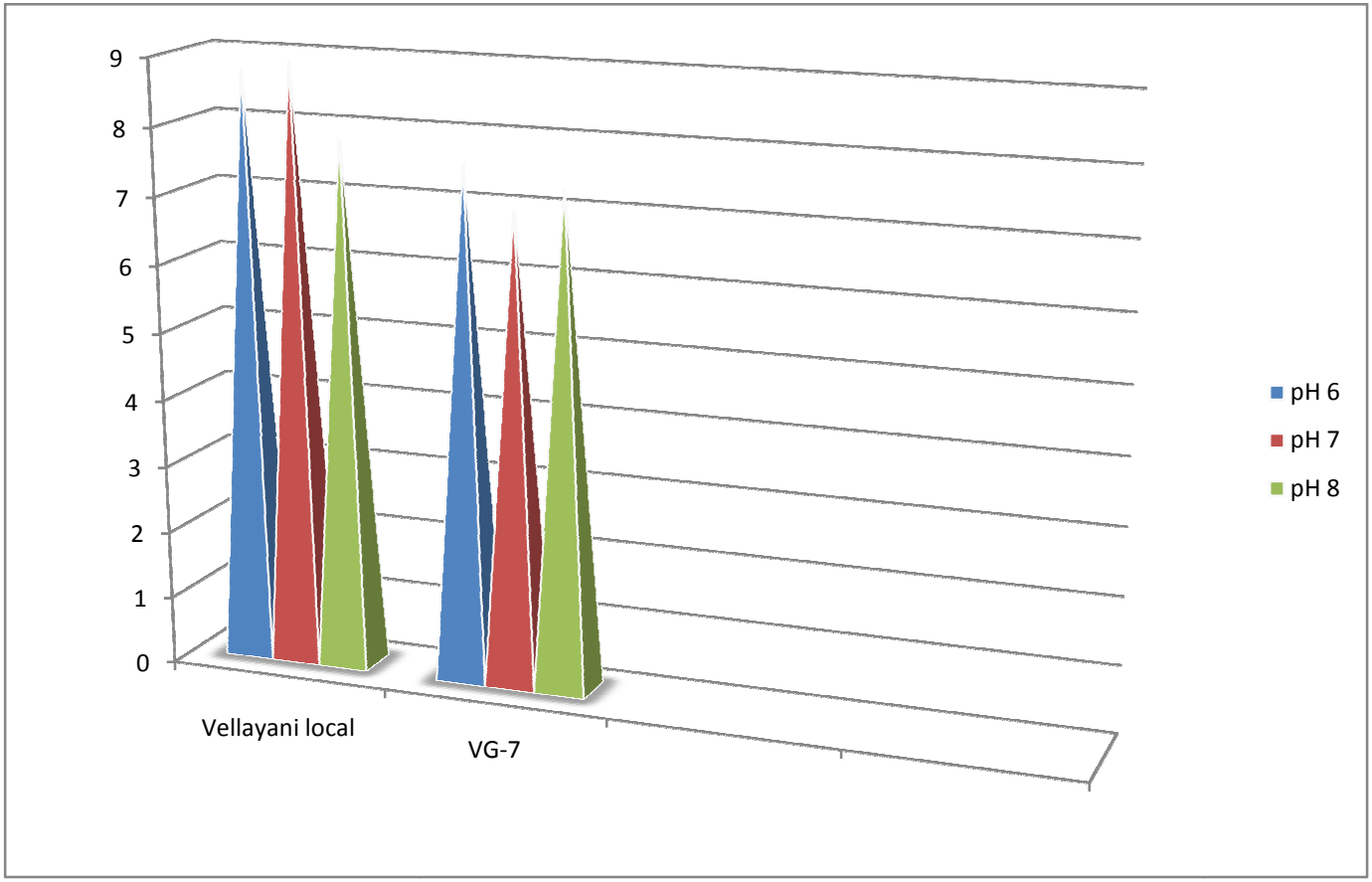


Fig. 2. Mycelial growth of the two *Ganoderma* isolates in different pH (in cm)

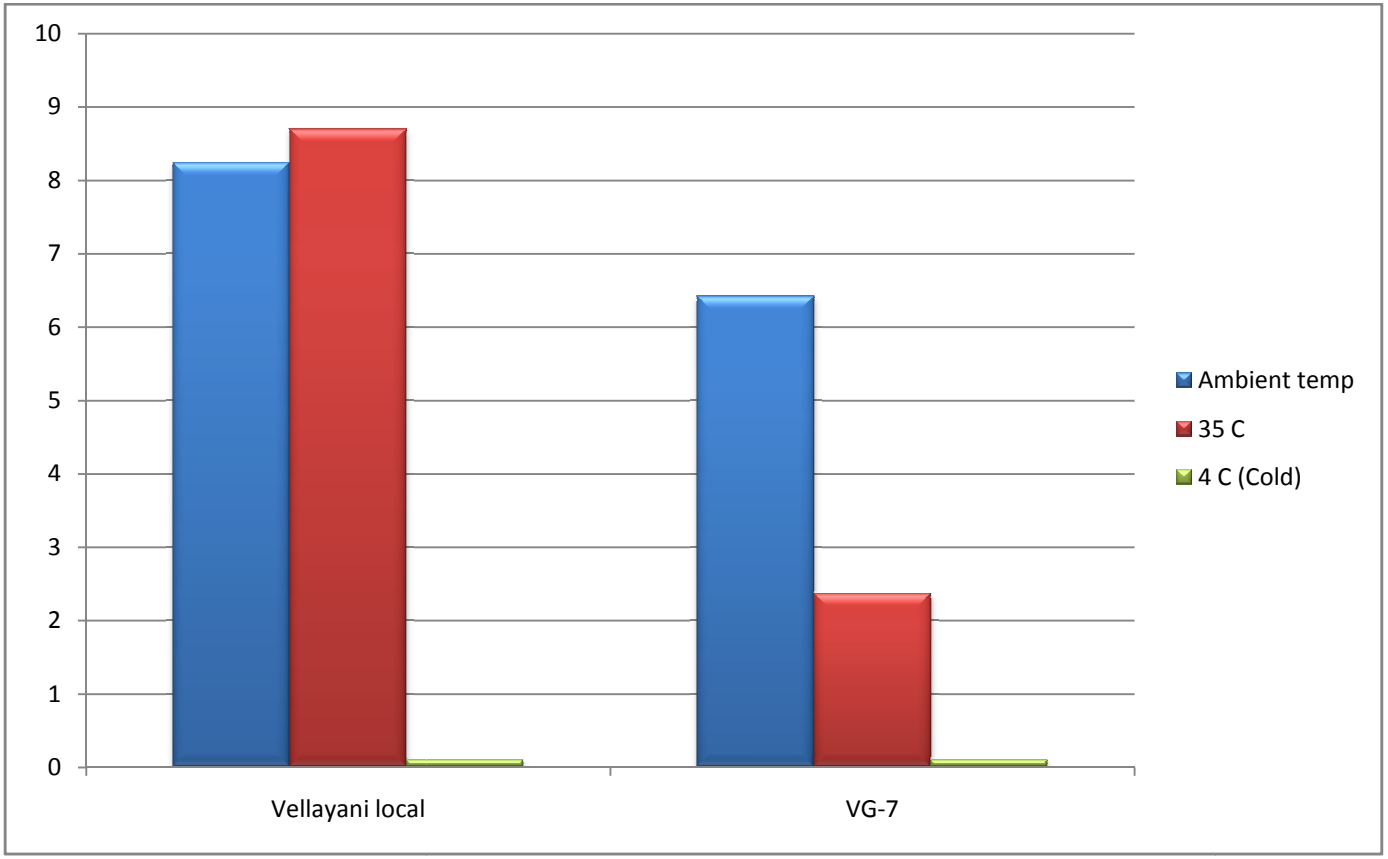


Fig. 3. Mycelial growth of the two *Ganoderma* isolates in different temperatures (in cm)

above 30ºC and below 20ºC while Jeong *et al.*, (2005) reported that the optimum temperature for the mycelial growth of *G. applanatum* was 25ºC and these results can be attributed to the fact that the *G. applanatum* is more suited to cooler temperature regimes.

The best spawn substrate selected based on minimum time taken for the spawn run and the nature of growth, was wheat followed by sorghum and paddy. Both of the isolates Vellayani local and VG-7 grew as thick white, fluffy mycelium all over the wheat grains within twelve to fifteen days. It took fifteen days for the fungus to colonise on sorghum grains and twenty days to colonise on paddy (Fig. 4). Veena and Pandey (2010) have reported the efficacy of sorghum as a base material for *Ganoderma* spawn production. Geetha and co-workers in 2012 have reported the suitability of paddy grains for the *Ganoderma* spawn production which is in agreement with the results obtained in the present study.

In the present study among the different sawdust based substrates tested, only sawdust and wheat bran combination provided favourable conditions for spawn growth. Also, rate of contamination was high when sawdust substrates were used. These materials took twenty one to thirty three days for complete mycelial colonization. Veena and Pandey (2010) have reported the unsuitability of sawdust based substrates for *Ganoderma* spawn production which is in line with the observations made in this study.

The culture Vellayani local took significantly shorter time for the complete mycelial colonization in the four substrate combinations consisting of sawdust and bran alone i.e.; sawdust (90%) + rice bran (10%), sawdust (90%) + wheat bran (10%), sawdust (80%) + rice bran (20%), sawdust (80%) + wheat bran (20%). The isolate Vellayani local took the shortest time of 18.33 days to entirely colonise the substrate sawdust (90%) + wheat bran (10%) which was on par with the substrate sawdust (90%) + rice bran (10%) in which it took 18.50 days. Therefore sawdust

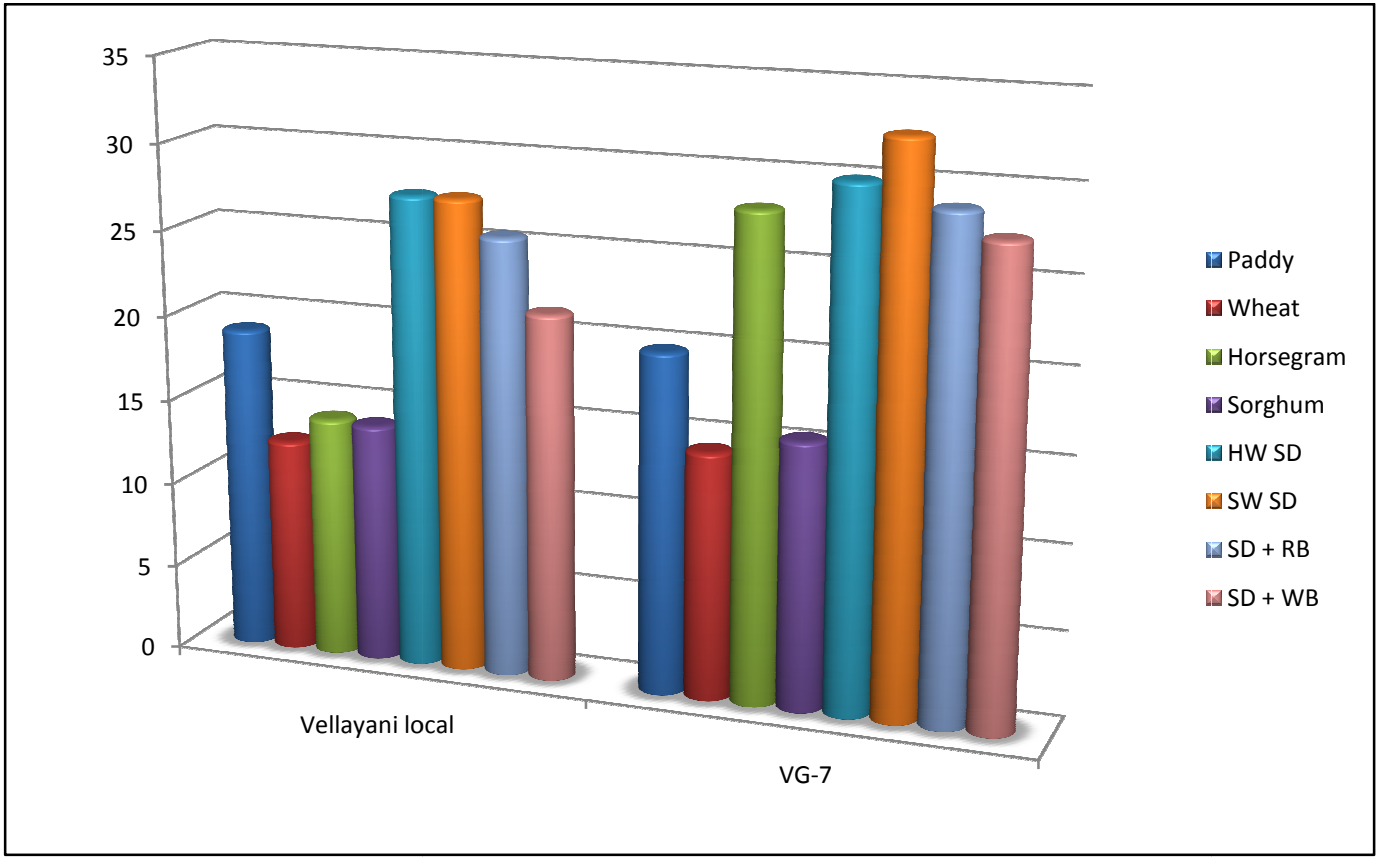


Fig. 4. Time taken for full colonisation of spawn substrates by cultures VG-7 and Vellayani local (days)

(90%) + rice bran (10%) is the best among the eight substrates evaluated for mycelial colonization of the fungus *Ganoderma*. Veena and Pandey (2006) have studied in detail the possibility of using sawdust and different types of spawn and found that sawdust (90%) with rice or wheat bran (10%), or sawdust (80%) with rice bran (18%), calcium carbonate (1%) and sucrose (1%) were the best substrates to support the cultivation of *Ganoderma* which is in consonance with the findings of the present study. Among the two isolates evaluated in the present study, Vellayani local took significantly shorter time for the primordial initiation in all the substrates. The two substrates containing wood chips did not develop any pinheads even after incubation for a month this could be attributed to the innate hardness of the substrate that will interfere with spawn run and further growth.

Among the two isolates, VG-7 recorded maximum yield on all the substrates evaluated. The highest yield of VG-7 was 27.41 g which was obtained in the substrate sawdust (80%) + rice bran (20%) followed by 25.32 g in sawdust (80%) + wheat bran (20%). These two results were on par with each other. For the isolate Vellayani local, highest yield recorded was 18.34 g in the substrate sawdust (80%) + wheat bran (20%) which was significantly different from the next highest yield of 15.76g obtained in sawdust (78%) + rice bran (20%) + $CaCO_3$ (2%). Among the two isolates, VG-7 recorded maximum biological efficiency in all the substrates evaluated Veena and Pandey (2006) have also observed the superiority of sawdust + bran substrates in mushroom cultivation which is in line with the findings of this study. Hossain and co-workers (2009) reported that there is considerable variation in the spawn run of *G.lucidum* on sawdust of different tree species.

The moisture content of *Ganoderma* was found to be 58.2% and 53.4% in Vellayani local and VG-7 respectively (fresh mushroom). Protein content present in the mushroom was estimated to be 20% while the fat content present in the mushroom was found to be 4.1% and 2.0% in Vellayani local and VG-7 respectively. Carbohydrate content present in *Ganoderma* mushroom was estimated by anthrone

method and it was found and expressed in percentage as 18.25% in Vellayani local and 20.50 in VG-7. The ash content present in the isolates of the mushroom was found to be 1% and 1.23% for Vellayani local and VG-7 respectively.

Hafiz and associates have also reported 16.9% protein in *Ganoderma* isolates as a part of their study on the cultivation of *G. resinaceum.* Tseng *et al*. (1984) have observed that *G. lucidum* has 4.6% fat content which is comparable to the fat level obtained in this study. These workers have also recorded carbohydrate 75%, soluble proteins 8.3% and total ash 3.62% and the discrepancy in the case of protein content can be attributed to the difference in methodology adopted.

In the submerged culture studies it was observed that a yield of 9.86 g and 10.01 g was obtained for the culture Vellayani local in carrot broth and oatmeal broth respectively while VG-7 yielded 11.83 g and 12.35 g respectively in carrot broth and oatmeal broth. Liquid or submerged culture has a potential advantage over solid media because it requires minimal space and there are less chances for contamination and the mycelium can be dispersed in the medium more uniformly (Bae *et al.*, 2000).

Summary

6. SUMMARY

Forest and homestead areas across the state were surveyed during the rainy season of 2011 and 2012 and 20 samples of *Ganoderma* fruiting bodies belonging to different stages of growth were collected. Surveys were mainly concentrated in Thiruvananthapuram district and the morphological characteristics of the collected mushrooms were recorded.

The collected mushrooms were subjected to tissue isolation. Three isolates of *Ganoderma* were obtained in pure culture and were named as VG-1, VG-3 and VG-7 and two isolates namely DMR-G and Vellayani local were procured from Directorate of Mushroom Research, Solan, HP and the Instructional farm, Vellayani respectively. The DNA of the three cultures was isolated and sequenced, their molecular characterization was done and the identity was confirmed and a phylogenetic tree was prepared.

All five isolates in potato dextrose agar (PDA) initially grew as white mycelia but the mycelial density varied with the cultures. While VG-1, VG-3 and VG-7 grew as thick white flocculent mycelia, Vellayani local and DMR-G isolates developed thin mycelia. The nature of growth also differed considerably. VG-3 and DMR-G initially grew in irregular patterns whereas the other cultures exhibited a circular pattern of growth. Among the cultures, Vellayani local was the fastest growing one which completed its *in vitro* growth in a week followed by the isolate VG-7 and so these cultures were selected for further studies. Microscopic characteristics like shape, size and colour of basidiospores and presence or absence of chlamydospores were also observed and recorded.

Five different nutrient media, *viz.*, potato dextrose agar (PDA), carrot agar (CA), rose bengal agar (RBA), oat meal agar (OMA) and *Ganoderma* extract agar

were evaluated for their efficacy in supporting the growth of the *Ganoderma* isolates in vitro. The colour and nature of growth of all the isolates were recorded by periodical visual observation. Carrot agar (CA) medium was found to be most suitable for the growth of *Ganoderma* spp. The mycelial growth was very low in *Ganoderma* extract agar and overall mycelial growth was poor in rose bengal agar (RBA).

Three different H^+ ion concentrations (pH) 6, 7 and 8 were evaluated for identifying the best pH condition that supports the growth of selected *Ganoderma* cultures. PDA was used for the experiment. The medium was prepared and the pH was adjusted to 6.0, 7.0 and 8.0 by adding 0.1 N hydrochloric acid (HCl) or 0.1 N sodium hydroxide (NaOH). Further studies revealed that a slightly acidic pH of 6 was best suited for the *in vitro* growth of *Ganoderma* spp.

Three different temperature conditions of 4ºC, ambient temperature and 35ºC were evaluated for the identification of best temperature suitable for the culture growth of *Ganoderma*. The result showed that different temperature conditions differ significantly in influencing radial growth and a temperature range of 30-35 ^{o}C was best suited for the *in vitro* growth of *Ganoderma* spp.

Three different light conditions of artificial light, normal light and darkness were tested for the efficacy in the production of maximum radial mycelial growth. The intensity of light had no significant effect on mycelial growth.

Eight different substrates including four grain and four sawdust based substrates were evaluated for their efficacy as spawning substrate. The sawdust of coconut was used as hardwood sawdust and the sawdust obtained from rubber was used as softwood sawdust. The grain substrates used in the experiment were paddy, sorghum, horse gram and wheat and the sawdust based substrates used include softwood sawdust, hardwood sawdust, sawdust combined with rice bran and sawdust combined with wheat bran. In the combination substrates, hardwood sawdust was

used. The best spawn substrate was selected based on the minimum time taken for the spawn run and the nature of growth.

Wheat grain was the best among the different spawn substrates tested since it took least time (13.50 days) for complete spawn run, followed by sorghum and paddy which completed the spawn run within 14.79 and 19.42 days respectively. Among the different sawdust based substrates tested, only sawdust and wheat bran combination provided favourable conditions for spawn growth. Also, rate of contamination was high when sawdust substrates were used. These materials took twenty one to thirty three days for complete mycelial colonization.

Eight different substrate combinations were used independently for evaluating their efficacy in cultivating the mushroom which included sawdust (90%) + rice bran (10%), sawdust (90%) + wheat bran (10%), wood chips (80%) + rice bran (20%), wood chips (80%) + wheat bran (20%), sawdust (80%) + rice bran (20%), sawdust (80%) + wheat bran (20%), sawdust (80%) + rice bran (18%) + $CaCO_3$ (1%) + sucrose (1%) and sawdust (78%) + rice bran (20%) + $CaCO_3$ (2%).

Among the bed substrates evaluated, sawdust (90%) + rice bran (10%) took the least time for complete mycelial colonization. The substrate sawdust (80%) + wheat bran (20%) was best suited for pinhead initiation of both the isolates. It was also observed that sawdust (78%) + rice bran (20%) + $CaCO_3$ (2%) was ideal for early fruiting of both strains of mushrooms, below 21 days. The substrates based on wood chips were not found effective for pinhead initiation and thus sporocarp production was not observed.

Among the cultures, total crop growth period was the least for Vellayani local whereas VG-7 recorded maximum yield in all the substrates evaluated. The highest yield of VG-7 was 27.41g which was obtained in the substrate sawdust (80%) + rice bran (20%) and the highest yield recorded for Vellayani local was 18.34g in the substrate sawdust (80%) + wheat bran (20%) on fresh weight basis.

Among the two isolates, VG-7 recorded maximum biological efficiency in all the substrates evaluated. The highest biological efficiency of VG-7 was 6.59% which was obtained in the substrate sawdust (80%) + rice bran (20%) and 4.41% for Vellayani local in the substrate sawdust (80%) + wheat bran (20%).

Submerged culture production was done in two liquid media namely carrot broth (CB) and oatmeal broth (OMB). Vellayani local yielded 9.86 g and 10.01 g in carrot broth and oatmeal broth respectively while VG-7 gave a yield of 11.83 g and 12.35 g in CB and OMB respectively per 100 ml medium used.

As part of the investigation, 20 species of *Ganoderma* was collected and described, Vellayani local was found to be the best *Ganoderma* culture based on in vitro growth; carrot agar was the most suited medium; an acidic pH and a temperature range of 30-35^{o}C favoured the growth; wheat was the most suitable spawning material; sawdust substrates amended with bran was the most efficient bed material and VG-7 was superior in terms of biological efficiency.

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Appendices

APPENDIX – I

DNA sequences of the three isolated *Ganoderma* cultures

VG1

AAGGATCATTAACGAGTTTTGAAACGGGTTGTAGCTGGCCTTCCGAGG
CATGTGCACGCCCTGCTCATCCACTCTTACCTCCTGTGCACTTACTGTA
GGCTTCAGGCGCGCTGCGGCTTTCAACGCCGTGACGTTACTGGGTTTA
CGTTTTACTACCAACTACAAAGTATCAGAATGTGTATTGCGATGTAAC
GCATCTATATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGAT
GAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAG
TGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAGG
AGCATGCCTGTTTGAGTGTCATGAAATTCTCAACCTACAAATCTTTGCG
GGTTTGTAGGCTTGGACTTGGAGGTTTTTGTTGGCTTCTCGCAAGTCGG
CTCCTCTTAAATGCATTAGCCTGTTTCCTTGCGGATCGGCTCTCGGTGT
GATAATTATCTGCGCCGCGACCGTGAAGCGTTTGGCTGGCTTCTAATC
GTCTCGCTCAAGAGACAGCTTCTTATGACCTCTGACCTCAAATCAGGT
AGGACTACCCGCTGAACTTAA

VG3

GGGTTGTAGCTGGCCTTCCGAGGCATGTGCACGCCCTGCTCAATCCAC
TCTACACCTGTGCACTTACTGTGGGTGACGGATCGCAAAGCGGGCTCT
TGTCCGTTATAAGCGCATCTGTGGCCTGCGTTTACCACAAACTCTTTGA
AAGTACTAGAATGTAATATTGGGATATAATAGATCTATATACAACTTT
CAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAAT
GCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTG
AACGCACCTTGCGCTCCTTGGTATTCCGAGGAGTATGCCTGTTTGAGTG
TCATGAAATCTTCAACTTGCAACCTCTTTGCGGAGTTTGTAGGCTTGGA
CTTGGAGGGCTTGTCGGCCTTTAATGGTCGGCTCCTCTTAAATGCATTA
GCTTGATTCCTTGCGGATCGGCTGTCGGTGTGATAAAATGTCTACGCC
GTGACCGTGAAGCGTTTGGATGAGCTTCCAACCGTCTTGCTTCAAAGA
CAAGTTTTATATGACCTCTGACCTCAAA

VG7-PARTIAL

GCTGGCCTTTCCGAGGCATCGTGCACGCCCTGCTCATCCACTCTACACC
TGTGCACTTACTGTGGGTTATGGATCGTGCGGAGCGGGCTCTTTGACG
AGTTTGCGAAGCGCGTCTGTGCCTGCGTTTT

APPENDIX – II

Composition of different media

a) Potato dextrose agar (PDA)

Potato	:	200 g
Dextrose	:	20 g
Agar-agar	:	20 g
Distilled water	:	1 l

b) Oat meal agar

Oats	:	40 g
Agar-agar	:	20 g
Distilled water	:	1 l

c) Carrot agar

Carrot	:	200 g
Dextrose	:	20 g
Agar-agar	:	20 g
Distilled water	:	1 l

d) *Ganoderma* extract agar

Ganoderma extract	:	200 ml
Dextrose	:	20 g
Agar-agar	:	20 g
Distilled water	:	1 l

Biology and Cultivation of *Ganoderma* spp.

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Abstract of the
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ABSTRACT

The present investigation on 'Biology and cultivation of *Ganoderma* spp.' was conducted at College of Agriculture, Vellayani, Thiruvananthapuram during the period 2011-2013. The aim of the experiment was to study the biology of indigenous species of *Ganoderma* and to standardize techniques for the cultivation of this medicinal mushroom.

Forest and homestead areas across the state were surveyed during the rainy season of 2011 and 2012 and 20 samples of *Ganoderma* fruiting bodies belonging to different stages of growth were collected. Three isolates of *Ganoderma* were obtained in pure culture and were named as VG-1, VG-3 and VG-7 and two isolates namely DMR-G and Vellayani local were procured from Directorate of Mushroom Research, Solan, HP and the Instructional farm, Vellayani respectively. The DNA of the three cultures was isolated and sequenced and molecular characterization was done and their identity was confirmed and a phylogenetic tree was prepared.

Among the cultures, Vellayani local was the fastest growing one which completed its *in vitro* growth in a week followed by the isolate VG-7 and so these cultures were selected for further studies.

Carrot agar (CA) medium was most suitable for the growth of *Ganoderma* spp. Physiological studies revealed that a slightly acidic pH of 6 and a temperature range of 30-35 ^{o}C were best suited for the in vitro growth of *Ganoderma* spp. The intensity of light had no significant effect on mycelial growth.

Wheat grain was the best among the different spawn substrates tested since it took least time (12 – 15 days) for complete spawn run, followed by sorghum and paddy which completed the spawn run within 16 and 20 days respectively.

Among the bed substrates evaluated, sawdust (90%) + rice bran (10%) took the least time for complete mycelial colonization. The substrate sawdust (80%) + wheat bran (20%) was best suited for pinhead initiation of both the isolates. It was

also observed that sawdust (78%) + rice bran (20%) + $CaCO_3$ (2%) was ideal for early fruiting of both strains of mushrooms, below 21 days. The substrates based on wood chips were not found effective for pinhead initiation and thus sporocarp production was not observed.

Among the cultures, total crop growth period was the least for Vellayani local whereas VG-7 recorded maximum yield in all the substrates evaluated. The highest yield of VG-7 was 27.41g which was obtained in the substrate sawdust (80%) + rice bran (20%) and the highest yield recorded for Vellayani local was 18.34g in the substrate sawdust (80%) + wheat bran (20%) on fresh weight basis.

Among the two isolates, VG-7 recorded maximum biological efficiency in all the substrates evaluated. The highest biological efficiency of VG-7 was 6.59% which was obtained in the substrate sawdust (80%) + rice bran (20%) and 4.41% for Vellayani local in the substrate sawdust (80%) + wheat bran (20%).

Submerged culture production was done in two liquid media namely carrot broth (CB) and oatmeal broth (OMB). Vellayani local yielded 9.86g and 10.01g in carrot broth and oatmeal broth respectively while VG-7 gave a yield of 11.83g and 12.35g in CB and OMB respectively per 100 ml medium used.

As part of the investigation, 20 species of *Ganoderma* was collected and described, Vellayani local was found to be the best *Ganoderma* culture based on in vitro growth; carrot agar was the most suited medium; an acidic pH and a temperature range of 30-35^{o}C favoured the growth; wheat was the most suitable spawning material; sawdust substrates amended with bran was the most efficient bed material and VG-7 was superior in terms of biological efficiency.