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Studying Soil Ecology and Growth Conditions of *Phellorinia herculeana*, a Wild Edible Mushroom

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Abstract: *Phellorinia herculeana* is an edible mushroom growing in nutritionally poor and desert soil. There has been little information available about its edaphic and culturing conditions for achieving the vigorous mycelial growth essential for its artificial cultivation, bioaugmentation and biodegradation in unfertile soil. Thus, the present study was conducted to assess its edaphic conditions and find a suitable culturing medium for obtaining maximum growth. It grows commonly in coastal soil with saline conditions, barren land soil unfit for cultivation, and desert soil. It forms a basidiocarp singly around xerophytic trees and annual plants and also in soil without vegetation. In addition to a well-developed pileus and stipe, it has a typical rhizoid that grows horizontally in soil. The rhizoid was thick at the base of the stipe and became thin into the mycelial strand. In our earlier study, we reported that its mycelial growth was very poor on nutrient-rich media containing simple sugar, for example, glucose. In the present study, we observed that cereal-grain-based agar media supported its mycelial growth and among the cereal-grain-based agar media, maize agar medium at the 5% level supported the maximum mycelial growth. Incorporation of glucose into the maize agar medium reduced its mycelial growth compared to its growth on maize agar medium without glucose. Its mycelial growth was at a maximum between 34 °C and 37 °C and at a pH between 7 and 8. Mass multiplication using sand-maize medium prepared at the ratio of 19:1 (sand: maize) supported the maximum mycelial growth. The results of this study would certainly pave a way for the scientific community to develop a protocol for its artificial cultivation and also for its mass multiplication, bioaugmentation and biodegradation in unfertile soil.

Keywords: fungus; barren land soil; coastal soil; desert soil; bioaugmentation

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1. Introduction

Phellorinia herculeana is a wild edible mushroom and artificial cultivation practices (domestication) have not been developed yet for its basidiocarp production. It is considered as a specialty mushroom because it is less common in a particular area or in a country. It comes under the family -*Phelloriniaceae* [1]. It is a gasteromycetes fungus since it has millions of basidiospores that are enclosed in the spherical-shaped basidiocarp. Though several humicolous mushrooms grow in soil containing high amounts of organic carbon content and clay-type soil with high water-holding capacity, *P. herculeana* grows in coarse sandy soils with low water-holding capacity and low organic carbon content. It grows in arid climatic conditions in regions of desert area, with xerophytic vegetation mainly consisting of

prosopis, acacia and cactus plants, and coastal area, having salty and sandy soil, in several parts of world such as India, Pakistan, Baluchistan, Argentina, Spain, etc. [2–6]. It appears sub-hypogeously at the top layer of the loose sandy soils during rainy periods [7]. It is also called the desert edible mushroom since it grows in arid and desert climatic conditions.

Extracts of *Phellorinia sp.* effectively inhibited several human bacterial pathogens, especially *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Staphyloccocus aureus* [8,9]. Though it is an edible mushroom with high nutraceutical and pharmaceutical properties, there is no study on finding suitable growth media, tested for culturing its mycelial biomass, and factors affecting its growth conditions. Its mycelial growth is favored by hot temperature at 35 °C and alkaline pH conditions [2]. To our knowledge, based on a literature search in PubMed® database at the NCBI (National Centre for Biotechnology Information), there is no peer-reviewed literature about *Phellorinia,* and there are only a few non-peer-reviewed studies on the Google Scholar database, describing mostly its phylogeny, physiology and morphological characters. In our previous study, we noticed that *P. herculeana* grew poorly on commonly used nutrient-rich culture media such as potato dextrose agar medium (PDA) and yeast extract-peptone-dextrose medium (YPD) [10]. Thus, further research work was carried out to gain knowledge on its ecology and the edaphic conditions required for its basidiocarp production and assess various growth media to obtain the maximum biomass. The results of this study would further help in developing a methodology for its domestication and bioaugmentation in unfertile soil.

2. Materials and Methods

2.1. Survey, Edaphic Conditions, Isolation and Tissue Culture of P. herculeana

Firstly, various edaphic conditions such as soil types, soil organic matter levels and types of vegetation at the habitat of *P. herculeana* were recorded [7]. Well-grown sporocarps were collected from soil at a nearby coastal region and kept on a sterile tissue paper. Sporocarps were surface-sterilized with 70% ethyl alcohol using absorbent cotton and were split longitudinally into two halves. Using a new sterilized blade, a small piece of plectenchymatous tissue was cut from the center of the split mushroom at the junction point of the pileus and stipe and inoculated onto the PDA medium amended with 100 µM of streptomycin sulphate (Ambistryn-S®, Abbott Healthcare, Chicago, IL, USA) to avoid bacterial contamination.

2.2. Assessment of Various Cereals and Pulse Grain-Based Agar Media for the Mycelial Growth of P. herculeana

Various commonly available cereal grains such as paddy, wheat, maize, sorghum, cumbu and oats, and pulse grains such as green gram, red gram and cowpea, were ground into fine powder. These powdered grains were dissolved separately in water at the 5% level and agar was added at the 2% level and autoclaved. After autoclaving, streptomycin sulphate was added to the culture media at 100 µM levels and poured into Petri dishes. The actively growing culture of *P. herculeana* was center-point inoculated and incubated in an incubator at 34 ± 2 °C under 10 h light and 14 h dark and aerated conditions for five to ten days [10]. Radial growth of the mycelium was recorded when the mycelial growth covered in any one of the culture media**.** Each treatment was replicated three times and the experiment was repeated twice.

2.3. Assessment of Various Concentration of Maize Grains and Glucose on the Mycelial Growth of P. herculeana

Maize agar medium was prepared at different concentration levels viz., 2.5, 5, 7.5 and 10% and autoclaved. After autoclaving, streptomycin sulphate was added to the culture media at 100 µM levels and poured into Petri dishes. The actively growing culture of *P. herculeana* was center-point-inoculated and incubated at 34 ± 2 °C. Radial growth of the mycelium was recorded when the mycelial growth covered any one of the culture media**.** Each treatment was replicated five times and the experiment was repeated twice.

The influence of glucose in cereal-grain-based agar medium was tested by incorporating glucose at 1 and 2% levels in maize agar medium (5%) and also maize agar medium alone without glucose for comparison. Inoculation of the culture and radial growth of the mycelium were recorded as described above. Each treatment was replicated seven times and the experiment was repeated twice.

2.4. Assessment of Various Temperature Conditions on the Mycelial Growth of P. herculeana

Five-millimeter culture discs were cut with a sterilized cork-borer from advancing margins of the colonies of fungus and inoculated on 5% maize agar medium supplemented with streptomycin sulphate (100 µM) at the center of the culture plate. The cultures were incubated at different temperature conditions viz., 30, 34, 37 and 40 °C. Radial growth of the mycelium was recorded as described above. Each treatment was replicated six times and the experiment was repeated twice.

2.5. Assessment of Various pH Conditions on the Mycelial Growth of P. herculeana

Maize agar medium (5%) was prepared in conical flask and pH of the medium was adjusted viz., 5.0, 6.0, 7.0, 8.0 and 9.0 separately with 0.1 N lactic acid or 0.1 N NaOH and sterilized in an autoclave. For preparing maize agar medium with pH 5.0 (and less than pH 5.0), the lactic acid was added to adjust the pH after sterilization of the medium because lactic acid hydrolyses the agar under strong acidic conditions at a higher temperature (while autoclaving), resulting in non-solidification of medium. Inoculation of the fungus, its incubation and measurement of radial growth were carried out as described above. Each treatment was replicated four times and the experiment was repeated twice.

2.6. Assessment of Different Concentration Levels of Sand-Maize Medium

Coarse sand collected from coastal land was mixed with maize powder at 9:1, 19:1, 29:1, 49:1 and 99:1 (Sand: maize). The sand-maize medium (100 g) was taken in 250 mL conical flasks and autoclave-sterilized [11]. The medium was inoculated with a culture disc of *P. herculeana* and incubated for 10 days and appearance of mycelial coverage was recorded based on the score (1–5 scale): 1 =< 10% of the medium covered with mycelium; 2 = 10 to 25% of the medium covered with mycelium; 3 = 25 to 50% of the medium covered with mycelium, 4 = 50–75% of the medium covered with mycelium and 5 => 75% of medium covered with mycelium.

3. Results

3.1. Ecology and Edaphic Conditions of P. herculeana

Basidiocarps of *P. herculeana* were found in dry and sandy soil near coastal-region and barren-land soil that were very coarse in texture and poor in organic matter and vegetation. Commonly growing vegetation around its habitat consists of *Prosopis juliflora*, *Acacia nilotica* and cactus (Figure 1A). Its mycelium colonizes soil as thread-like strands. It forms a basidiocarp semi-hypogeously. The basidiocarp arises singly from the soil in the early morning. The basidiocarp contains pileus (cap), stipe without volva and annulus. It has a long and thick rhizoid. The pileus is globular or pyriform in shape and completely enclosed. The pileus has a thick outer mycelial mat consisting of plectenchymatous tissue-forming peridium and contains numerous spores adhering together inside the peridium. The stipe is cylindrical in form and almost half of its length lies in the soil (hypogeous) (Figure 1B,C). The rhizoid is formed at the base of the stipe at the thickness of 5–10 mm diameter and it becomes thin towards its end as plant roots. The rhizoid ultimately ends at the mycelial strands, which are visible for 60 to 90 cm length distance, and thereafter the mycelial strand is not visible. The basidiocarp is white in color and soft in texture and pleasant in taste upon cooking. The plectenchymatous tissue was isolated from the pileus and inoculated onto the PDA medium. The culture was incubated until it grew well and sub-cultured for the further study. In our previous study, we identified this unknown mushroom as *P. herculeana* by internal transcribed spacer sequence (ITS) analysis [10].

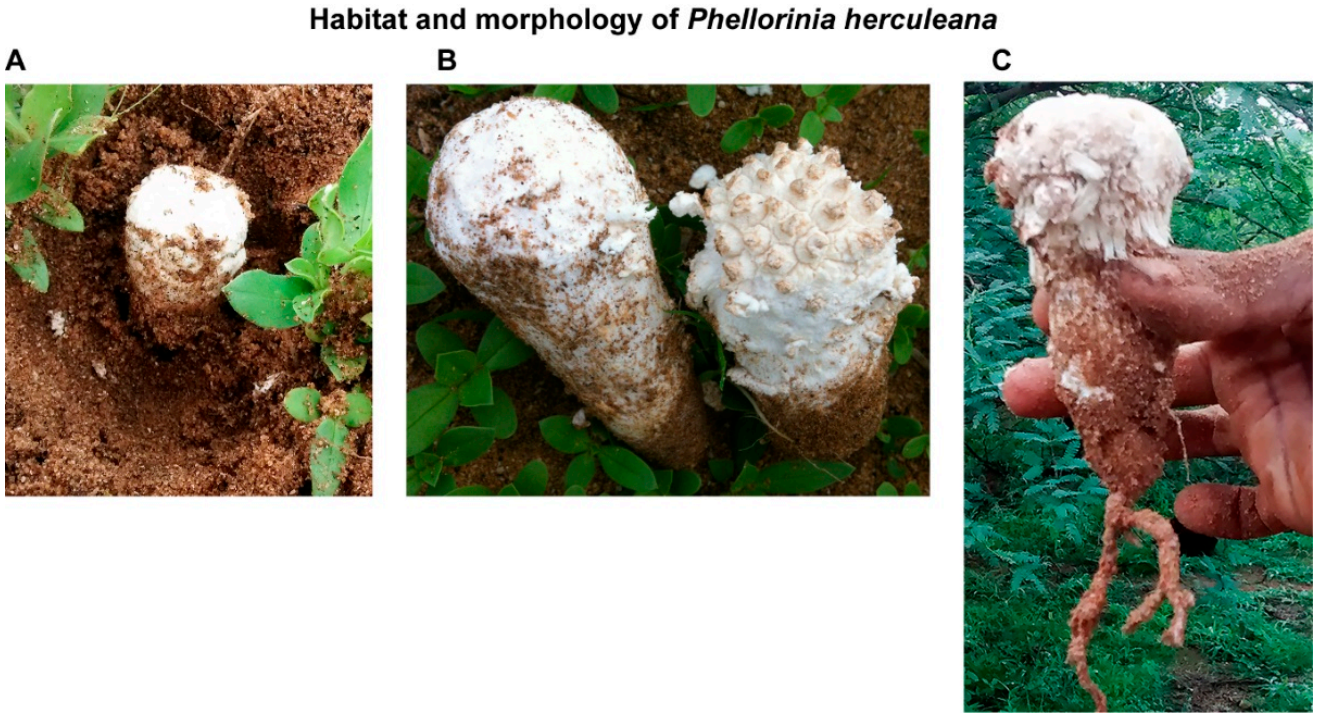

Figure 1. Habitat and morphology of *Phellorinia herculeana*. (**A**): Basidiocarp of *P. herculeana* grows sub-hypogeously in coarse sandy soil; (**B**): Pileus of *P. herculeana* appears as pyriform shaped; (**C**): Basidiocarp has pileus, stipe without volva and annulus and rhizoid.

3.2. P. herculeana Grows Well on Cereal Grains-Based Culture Media

In order to find the best substrate for culturing the *Phellorinia*, we used cereal grains and pulse grains in the culture medium. Cereal-grain-based agar media supported faster mycelial growth compared to pulse-grain-based agar media, as viewed based on the growth on the Petri plate (Figure 2A) and also measured quantitatively (Figure 2B).

Among the various cereal-grain-based media and pulse-grain-based media tested, maize medium showed the maximum mycelial growth (89.66 mm), followed by the rice medium and sorghum medium (89.33 mm mycelial growth), wheat medium (87.66 mm mycelial growth), cumbu medium (87 mm mycelial growth), cowpea medium (57.33 mm mycelial growth) green gram medium (53.66 mm mycelial growth) and red gram medium (50 mm mycelial growth). The least mycelial growth was observed in oat medium with 40 mm mycelial growth (Figure 2B). This study showed that cereal-grain-based media are generally good for its culturing.

Since *Phellorinia* produces sporocarps in soils poor in organic matter, we wanted to test what level/concentration of maize grain powder would be the optimum level for the growth and multiplication of *Phellorinia*. Thus, maize kernel powder was used at 2.5%, 5%, 7.5% and 10% concentration levels to assess the suitable concentration of maize grains in the growth medium. The maize medium containing 5% of maize kernel powder recorded the highest mycelia growth (88.6 mm) among the various concentration levels of maize kernel powder tested (Figure 3A,C). Maize medium containing 7.5% maize kernel power and 10% maize kernel powder recorded 84.4 mm and 75.4 mm mycelial growth, respectively. The least mycelial growth (37.2 mm) was observed in maize medium containing 2.5% maize kernel powder (Figure 3A,C). This study indicated that maize medium with 5% maize kernel power was the optimum concentration for its mycelial growth. Further studies were conducted using maize medium containing 5% maize powder.

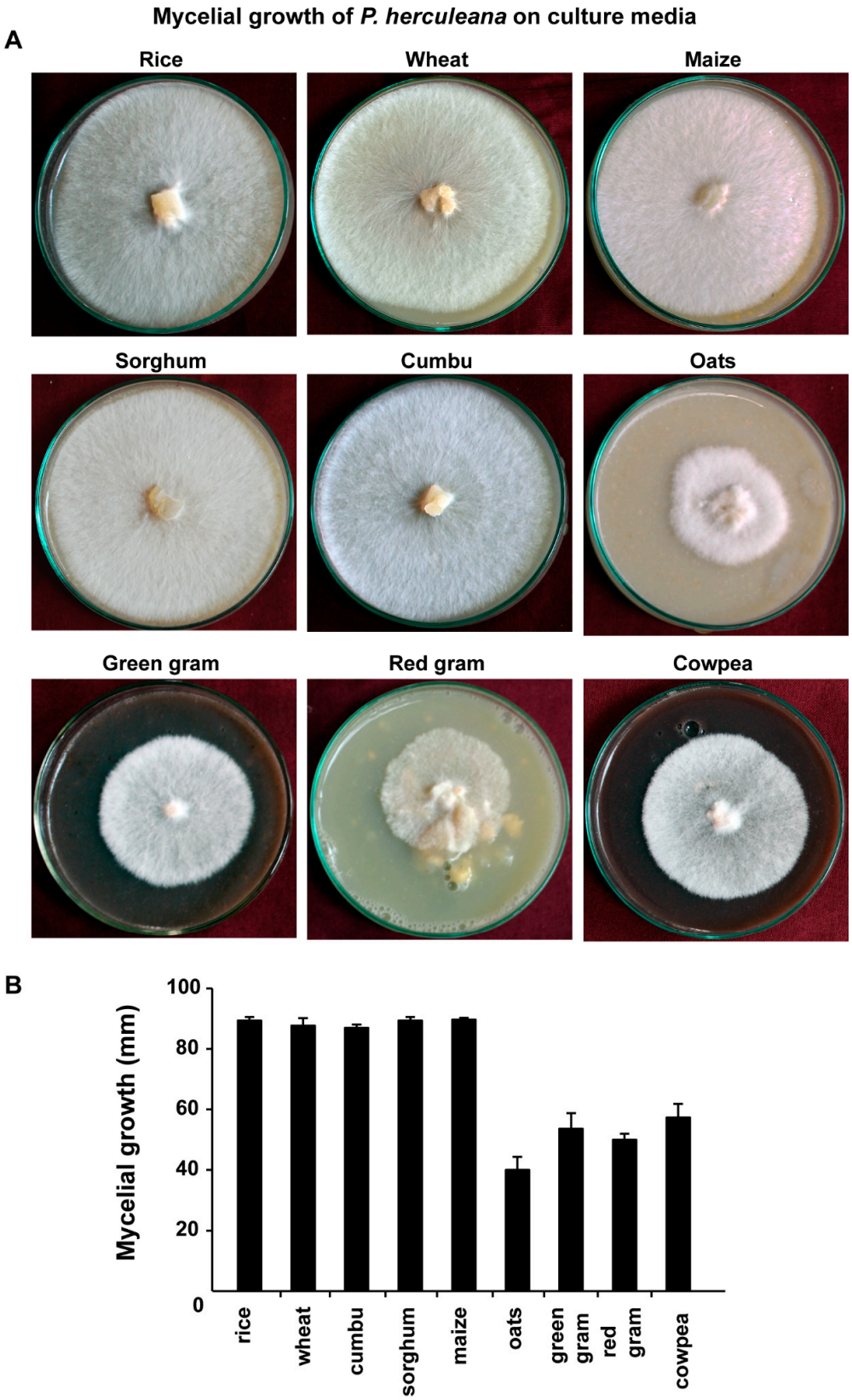

Figure 2. Mycelial growth of *Phellorinia herculeana* on culture media. (**A**): Mycelial growth of *P. herculeana* on cereal grain-based and pulse grain-based agar medium; (**B**): The radial growth of *P. herculeana*, grown on different culture media, was measured in millimeter. Error bar indicates the standard deviations.

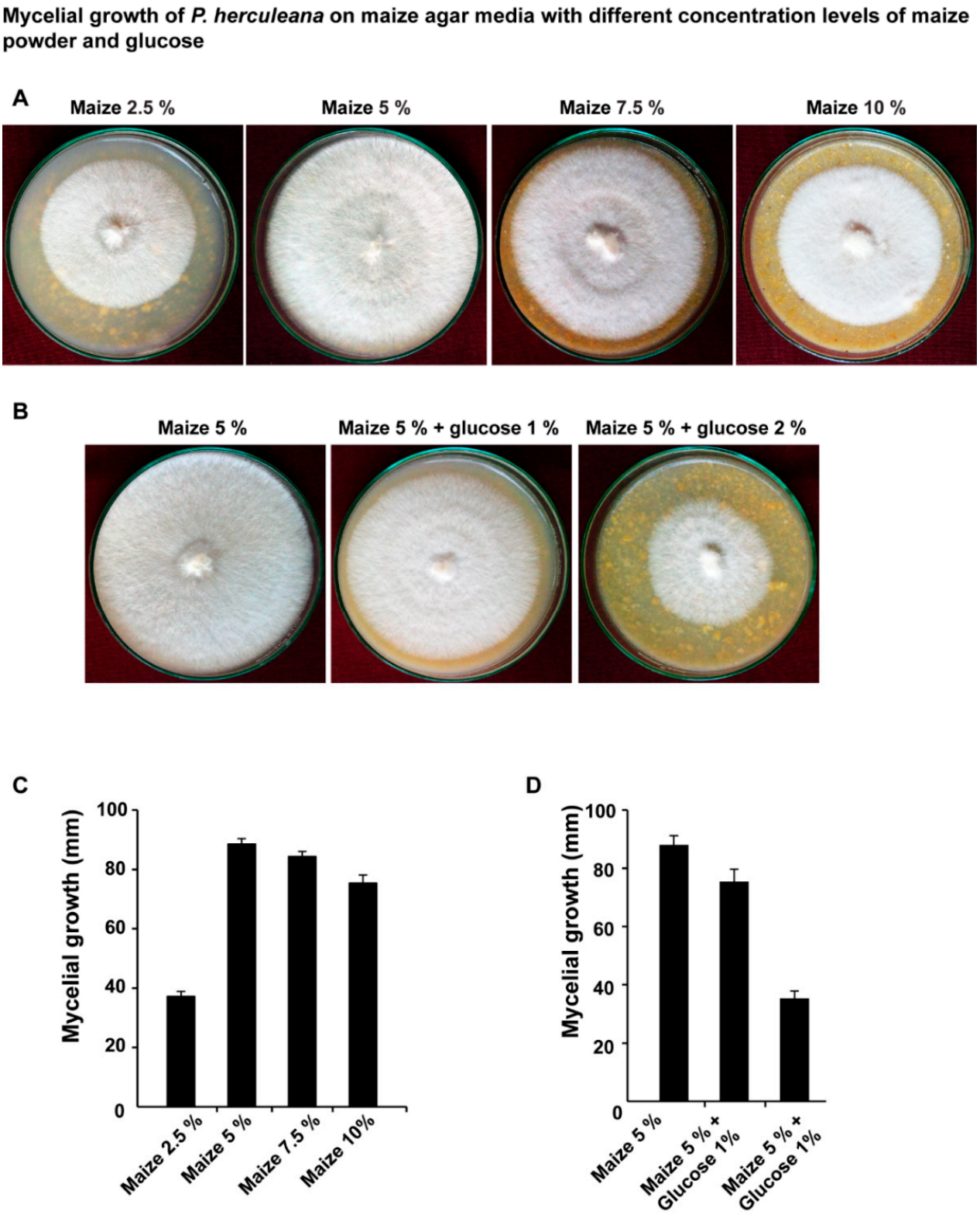

Figure 3. Mycelial growth of *Phellorinia herculeana* on maize agar media with different concentration levels of maize powder and glucose. (**A**): Mycelial growth of *P. herculeana* on maize agar medium at different concentration levels of maize powder; (**B**): Mycelial growth of *P. herculeana* on 5% maize agar medium incorporated with glucose; (**C**): The radial growth of *P. herculeana*, grown on maize agar medium with different concentration levels of maize powder, was measured in millimeter. Error bar indicates the standard deviations; (**D**): The radial growth of *P. herculeana*, grown on 5% maize agar medium incorporated with glucose, was measured in millimeter. Error bar indicates the standard deviations.

In our previous study, we noticed that *P. herculeana* grew very slowly on commonly used culture media such as PDA and YPDA containing glucose. Also, with regard to fruiting-body formation under natural conditions, its production was noticed in soils with low organic carbon content. We wondered if glucose has negative effect on its growth and differentiation. In order to make sure glucose has a negative effect on its growth, we tested its radial growth on the maize medium by incorporating glucose at different levels, such as 1 and 2% levels. As expected, the mycelial growth was reduced significantly at 1 and 2% glucose levels. The maize medium without glucose supported the maximum mycelial growth (88 mm). However, maize medium with 1% and 2% glucose levels showed the reduced mycelial growth (75.42 mm) and the least mycelial growth (35.28 mm), respectively. Thus, this experiment clearly showed that glucose has a negative effect on its mycelial growth on maize agar medium (Figure 3B,D).

3.3. P. herculeana Grows Well in Warm and Hot Temperature

The most critical and important physical factor affecting the mycelial growth in culturing mushroom fungi is temperature. Thus, to assess the optimum temperature for the culturing of *P. herculeana*, its mycelial disc was inoculated on maize agar medium and incubated at different growth temperatures viz., 30, 34, 37 and 40 °C, and radial growth of the mycelium was recorded. The maximum mycelial growth was observed when it was cultured at 34 °C (84.8 mm mycelial growth), followed by 37 °C (83.8 mm mycelial growth). The mycelial growth was reduced at 30 °C (48.4 mm mycelial growth). The very least mycelial growth (19 mm) was noticed at 40 °C. Thus, the present study shows that *P. herculeana* grows well in warm and hot temperatures between 34 and 37 °C. The conditions below 30 and above 40 °C would be detrimental for its growth (Figure 4A,B).

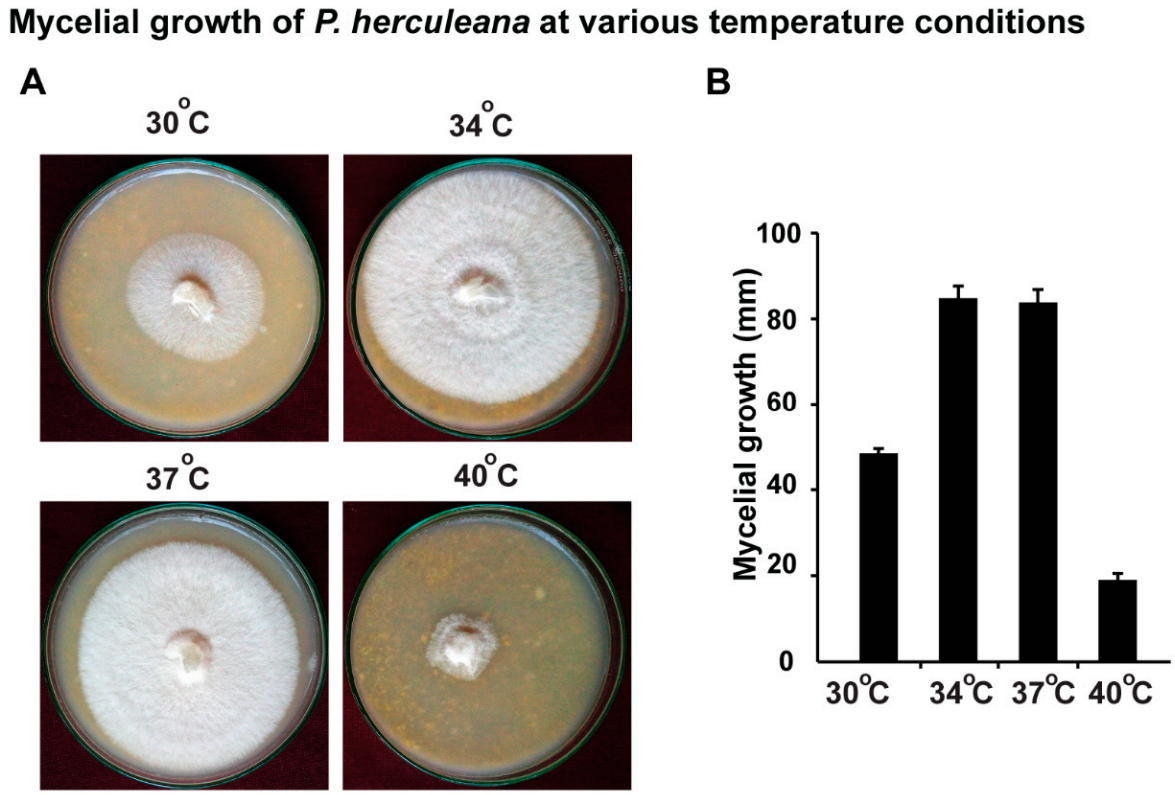

Figure 4. Mycelial growth of *Phellorinia herculeana* at various temperature conditions. (**A**): Mycelial growth of *P. herculeana* on maize agar medium incubated at different temperature conditions; (**B**): The radial growth of *P. herculeana*, grown at different temperature conditions, was measured in millimeter. Error bar indicates the standard deviations.

3.4. P. herculeana Grows Well at Slightly Alkaline pH

Each mushroom species requires the optimal pH range for its mycelial growth. We used maize agar medium with different pH levels viz., 5, 6, 7, 8 and 9. The results showed that the maximum mycelial growth (85.50 mm) was observed at pH 8.0, followed by pH 7.0, which supported 82.25 mm mycelial growth. *P. herculeana* also grew moderately at pH 9.0 with 77.25 mm mycelial growth. However, its growth was drastically reduced when the pH of the culture medium was reduced below neutral conditions. The maize medium at pH 6.0 showed reduced mycelial growth (66.75 mm). The mycelial growth is strongly reduced at pH 5.0, recording the least mycelial growth (13.75 mm). Thus, the present study shows that *P. herculeana* grows well at slightly alkaline pH conditions of pH 8 (Figure 5A,B).

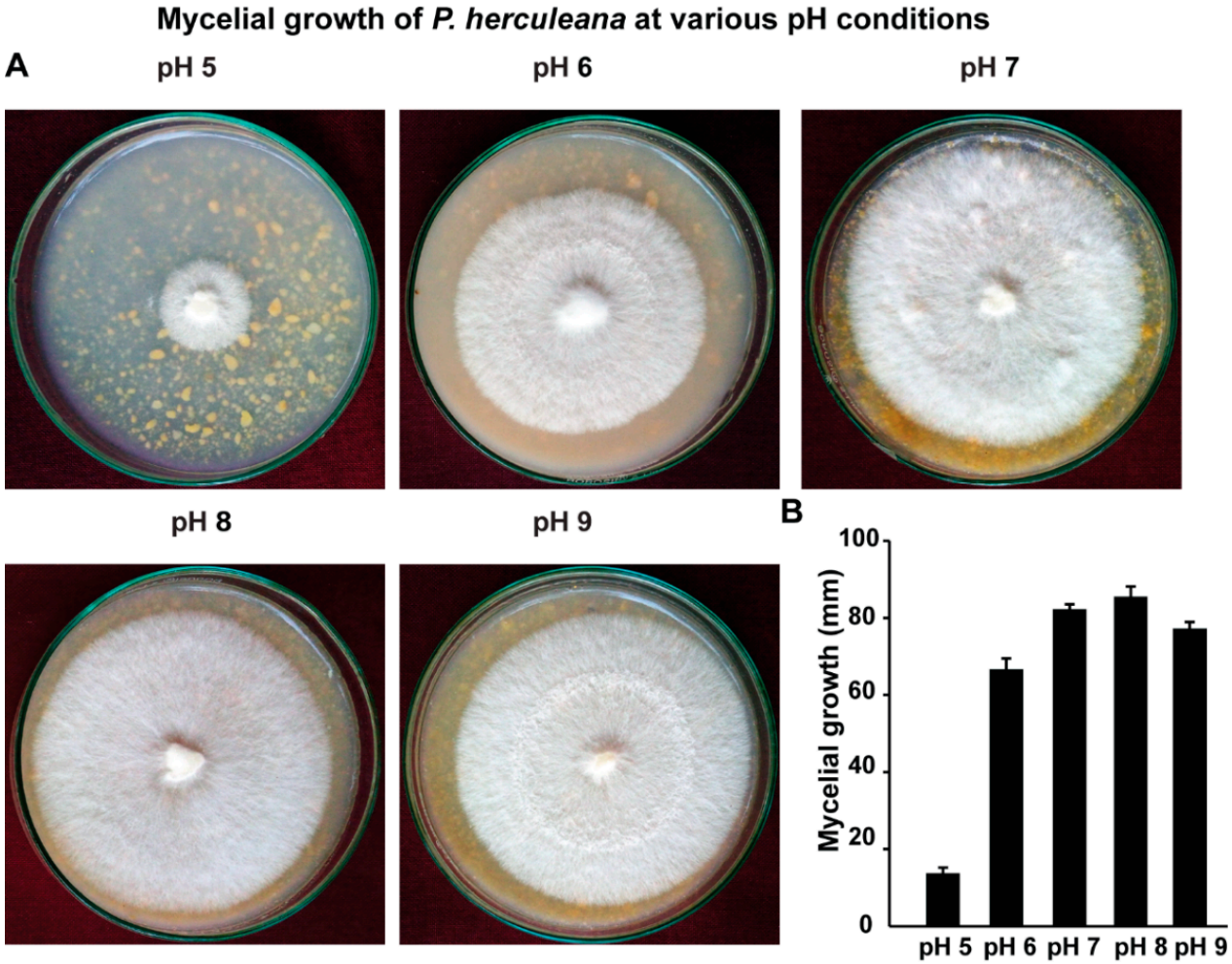

Figure 5. Mycelial growth of *Phellorinia herculeana* at various pH conditions. (**A**): Mycelial growth of *P. herculeana* on maize agar medium prepared at different pH conditions. (**B**): The radial growth of *P. herculeana*, grown at different pH conditions, was measured in millimeter. Error bar indicates the standard deviations.

3.5. P. herculeana Grows Well in Sand-Maize Medium at 19:1 Ratio Concentration

Among the various proportions of sand-maize media tested, the maximum mycelial growth was recorded in sand-maize medium at a 19:1 ratio and covered more than 75% of the sand-maize medium. Whereas the least the mycelial growth was seen in the sand-maize medium at a 99:1 concentration with mycelial growth covering between 10 to 25% of the sand-maize medium (Figure 6).

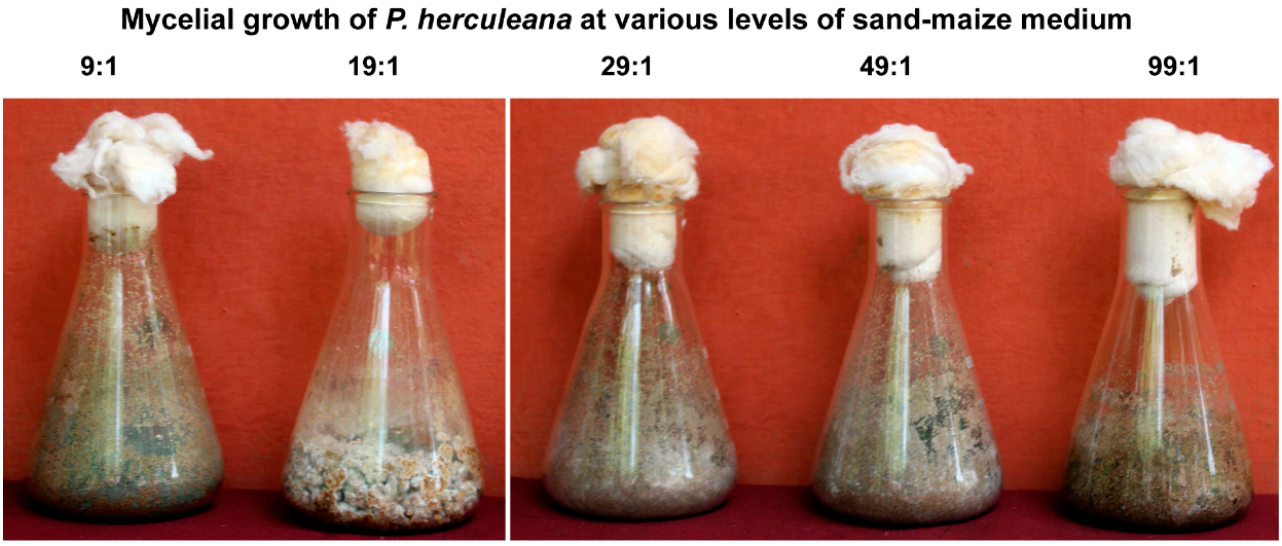

Figure 6. Mycelial growth of *Phellorinia herculeana* at various levels of sand-maize medium. Sand-maize medium was prepared with different levels of maize powder; inoculated with mycelial disc of *P. herculeana* and mycelial growth was recorded.

4. Discussion

For many years, local people in Tamil Nadu, India collected *P. herculeana* for edible purpose. Based on a literature search and our knowledge, the artificial cultivation practices for the cultivation of this mushroom has not yet been developed. It was noticed that this mushroom was found to have medicinal properties, nutritional value and delicious taste with pleasant appearance. Thus, domestication and commercialization of this mushroom would be advantageous for human society. In addition, its mass multiplication and bioaugmentation would improve the fertility of unfertile soil. Since there is no detailed study with regard to its artificial culturing using suitable culture medium and conditions influencing its growth and biomass production, there is a need to assess various growth conditions for culturing it on tissue culture media.

Several humicolous mushrooms usually grow on humus-rich soil containing high organic carbon and with high water-holding capacity. We noticed that *P. herculeana* grew typically in coarse sandy soil and land area where xerophytic plants such as prosopis, cactus, etc., usually grow. It also grows in sandy soil sown with millet crops such as sorghum and maize. The fruiting body has a thick rhizoid and tapers into a thin strand towards its end, as in crop plants. The tapered end of the rhizoid continues with thick strands of mycelium, which run into the soil horizontally towards the roots of the nearby growing tree/crop plants. Mycelial strands can be seen up to 60 cm length from the basidiocarp and thereafter the mycelial strand becomes thinner and thinner and cannot be visible. Thus, we speculate that because of the presence of the long rhizoid and mycelial strands, it can form either a parasitic or symbiotic relationship with other microorganisms in the soil or with the roots of the tree/crop plants as a mycorrhizal association, in order to obtain certain essential nutrients for its fructification. Similar edaphic conditions such as desert soil type with poor water-holding capacity and low organic carbon content have been observed for basidiocarp production of *Phellorinia* in a previous study [7]. Similarly, *Podaxis pistillaris* grows well in similar edaphic conditions in which *P. herculiana* does [7,12,13]. Both these wild edible mushrooms are seen in desert regions and collected by the local rural people as a source of income [14,15]. Interestingly, *P. pistillaris* grows in termite mounds in association with termites, in a symbiotic relation [13,16]. *P. herculeana* (previously reported as *P. inquinans*) was collected from the desert region in Australia [17], Pakistan [18], Kazakhstan [19] and an arid region of Yemen [20], and it is known for its gastronomical and nutraceutical value.

Initially, we attempted to grow and culture the mycelium of *P. herculeana* using commonly used culture media such as PDA, YPDA, malt extract medium, LB medium, etc. and

the mycelial growth was very slow on these media. Interestingly, it grew well in the soil extract medium that was prepared from the soil samples collected in its habitat. The habitat soil is a sandy type with low organic matter [10]. However, the mycelial growth on soil extract medium is sparse and low in biomass. For its mass multiplication, bioaugmentation and domestication, suitable culture medium should be identified that supports fast and thick mycelial growth. Several mushrooms are cultured on cereal-grain-based culture media. Thus, in the present study, cereal-grain-based agar media and pulse-grain-based agar media were evaluated. Cereal-grain-based agar media supported good mycelial growth compared to the pulse-grain-based agar media. Among the cereal-grain-based agar media, maize agar medium supported well at the 5% level. In our previous study, it was noticed that the mycelial growth was slow in media containing glucose, such as PDA and YPDA. We thought that simple sugar could reduce its mycelial growth. Thus, we evaluated the influence of glucose on its mycelial growth on maize agar medium. As expected, the incorporation of glucose reduced the mycelial growth on maize agar medium. Thus, from this study, we concluded that *P. herculeana* grows well on maize agar medium at 5% concentration levels. Adding simple sugar, especially glucose, retards the mycelial growth of *P. herculeana*. This result is intriguing because fungi generally prefer simple sugar for their growth. Due to the inhibitory effect of simple sugar, a few earlier studies could not succeed in its culturing [2,10]. We speculate that *P. herculeana* could secrete various cell-wall-degrading enzymes, break the complex polysaccharides and utilize the intermediatory products as a growth factors. Thus, the incorporation of glucose as an additive reduces its mycelial growth.

Temperature is a very important environmental factor for the mycelial growth of fungi. Each mushroom species requires a specific temperature for its optimum growth. Similarly, mushroom species generally grow in neutral or slightly alkaline pH conditions. *P. herculeana* grows well at hot temperatures between 34 and 37 °C and in slightly alkaline conditions. Lower temperature and lower pH conditions are not suitable for its growth. Similar to this present work, a previous study also showed that *P. herculeana* grew to the maximum level at 34 °C and grew well in a range of 30 to 40 °C [2], whereas there was no growth at 20 °C. It grew well at pH 6.6. Similarly, another study also showed that it grew at a hot temperature of around 40 °C and at pH 7.5 [21]. Its ability to form a mycelial mat and produce a basidiocarp under hot weather conditions in barren soil/desert soil/poor unfertile soil/coastal saline soil can be harnessed for its introduction in various parts of desert regions around the world, especially in the tropical regions.

Certain fungi such as mushrooms are well known for nutraceuticals such as proteins, minerals, and vitamins, and other fungi such as *Trichoderma* spp., *Aspergillus* spp., etc. produce both beneficial and detrimental compounds such as toxins [22–25]. *P. herculeana* is well known for its edibility around the world. However, it is an underexploited mushroom species because of its lack of artificial cultivation. We tried unsuccessfully to cultivate *P. herculeana* using various substrates, including the use of habitat soil as a substrate, under laboratory conditions. Hence, it is speculated that it could have mycorrhizal associations with plants or symbiotic associations with other soil biota. Due to this, it could not be artificially cultivated. However, the various findings of the present study, such as its edaphic conditions and optimum growth conditions, would be useful for enhancing its yield performance under its natural eco-system.

5. Conclusions

Since *P. herculeana* is an edible fungus, its domestication is a source of income. In addition, its bioaugmentation would be useful for the biodegradation of plant debris and improving the fertility of unfertile soil. Thus, the study on assessing its edaphic conditions and suitable growth conditions would help for its mass multiplication and bioaugmentation in barren land soil, leading to the improvement of soil productivity. In conclusion, this study clearly depicted that *P. herculeana* forms basidiocarps in coarse sandy-type soil around the roots of xerophytic trees, shrubs and millet crops under nutritionally

poor conditions, possibly by mycorrhizal association. Thus, it has good adaptations to extreme biotic and abiotic environments. It can be artificially cultured well on cereal-grain-based agar medium at 5% concentration levels at 34–37 °C and at a pH between 7 and 8. This information should be borne in mind when designing the protocol for its artificial cultivation and bioaugmentation.

Author Contributions: Conceptualization, V.R. and R.O.; methodology, R.O., G.S. and P.A.; software, R.O.; validation, R.O. and V.R.; formal analysis, K.M., V.S. and A.V.; investigation, V.R.; resources, V.R. and S.M.; data curation, N.R., P.M. (Palani Mahalakshmi) and P.M. (Petchimuthu Mareeswari); writing—original draft preparation, R.O.; Writing—review editing, R.O.; visualization, R.O.; supervision, V.R.; project administration, V.R. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest. All the experiments undertaken in this study comply with the current laws of the countries where they were performed.

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