Chapter 3
Materials and Methods

Abstract The chapter is divided into five sections which include: the need to collect these fungi, their habitat, procedures to collect them, their processing for further action. The last two sections deal with the procedures to isolate them *in vitro* and their preservation and the examination of the specimens for identification. The prerequisites for the collection and Do's and Do not's concerning field study are discussed in brief. The various procedures for *in vitro* isolation and preservation are also given.

3.1 Why to Collect

Wood-rotting non-gilled Agaricomycetes have drawn the attention of systematic mycologists as well as biotechnologists worldwide due to their ability to cause wood decay as well as for their application as tools in biotechnological processes. In the context of the Indian scenario; particularly the Himalayas; not much attention has been paid on the taxonomy and systematic of these fungi except for the work done only at the selected research laboratories located at Departments of Botany Panjab University, Punjabi University, Calcutta University and at the regional stations of Botanical Survey of India and at Mycology Herbarium at Forest Research Institute, Dehra Dun. No major initiative has been taken for their in vitro conservation. Since, The Himalayas are one of the mega biodiversity hotspots, attention must be paid for extensive exploration of these fungi for floristic analysis, for their ability to degrade wood and cause diseases of forest trees and their sustainable use in biotechnological processes.

3.2 Where to Collect

These fungi have a wide spread distribution in the forest ecosystem. They are found growing luxuriantly on fallen logs and twigs in the forests as well as elsewhere, where there is sufficient moisture available for their growth. A large number of these

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are found growing, producing their fructification on the living trees causing the heart-rot. Brown rot fungi are mostly confined to coniferous forests, where as the others are found growing on angiospermous trees or wood, causing decay. Fructifications of the larger fungi; which are generally pileate; are more prominent and easy to find on the tree trunk and branches of the trees, exposed surface of the fallen logs and on other exposed woody substrates than the resupinate fungi, which normally grow on the underside of the fallen logs, twigs or on the shady side of the parts of the trees. The underside of the decayed and rotten twigs on the forest floor are one of the richest sources of these fungi. Fallen twigs near the bank of the aquatic bodies including streams and rivulets also abound with the effused fructifications of these fungi. There is a marked difference between the flora of these fungi occurring on the trees and at their base from these found on decaying wood near the aquatic ecosystem. Some of these, like species of *Ganoderma* are particularly confined to the lower part of the tree trunk near the ground level, often forming a collar like growth as in case of *G. applanatum* (Fig. 3.1).

Some times during the rainy season especially during the monsoons, only the anamorphic stages of these fungi are found exclusively growing on specific trees. *Ptychogaster aurantiacus* Pat. (Fig. 3.2a), the anamorphic stage of *Laetiporus sulphureus* (Fr) Murr. (Fig. 3.2b) grows extensively on *Mangifera indica* in the foothills of N. W. Himalayas.

Critical observations are required to arrive at correct identification in such cases. Brown rot fungi have been found growing on man-made wooden infrastructure as doors, cabinets and other wood fixtures and cause enormous damage.

Fig. 3.1 *Ganoderma applanatum*

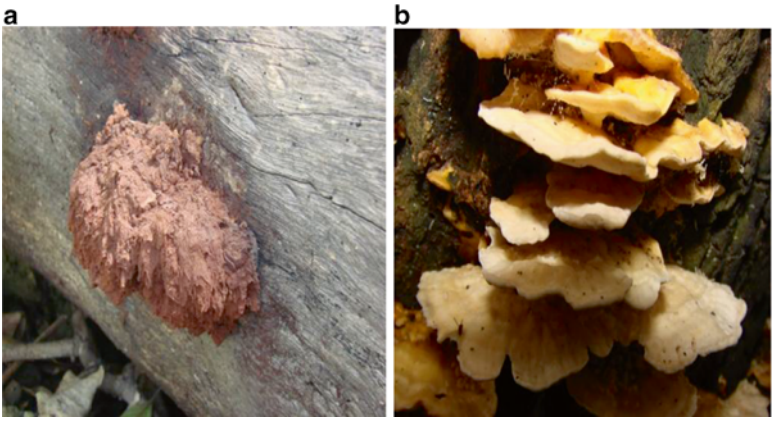

Fig. 3.2 (**a**) *Ptycogastor aurantiacus* (**b**) *Laetiporus sulphureus*

3.3 When and How to Collect

3.3.1 Collection Period

The most favourable season for making fresh collections of these fungi is in the monsoons, from the months of July to end of September. In some of the regions where the temperature does not fall much the collections of these fungi can be extended up to the mid of October. Although these fungi are found growing on wooden substrata throughout the year, these months are suitable for the collection of fertile and developing fructifications as well as collecting the mature ones with freshly developed hymenophore. The specimens collected are devoid of spores and hymenium during the summer months when the temperature is high with low moisture content and in the winters from mid October to end of February when the temperature is below normal for the growth of these fungi. Due to this reason most of the fungal forays particularly concerning these fungi are organized in the monsoon months and up to mid October. However, these fungi especially the resupinate ones are found growing on their substrate throughout the year in the microclimatic niches like shady places with sufficient moisture in the forest ecosystem in the himalayas, which favour their growth to some extent in the unfavourable months. There is a great variation in flora of these fungi during the pre-monsoon and in the winter months. Due to this reason, one or two extensive floristic surveys have to be conducted during these otherwise unfavourable periods of the year.

3.3.2 Procedure of Collection

3.3.2.1 Pre-requisites for Collection

Before moving out for field collection, the collector should check the following pre-requisite for the field collection. These include:

(i) Photo identity card from the employer (if one is working in a research/ Academic Institute along with an authorization to collect these fungi). This is essential since the regulation concerning conservation and protection of biodiversity has been issued by Ministry of Environment & Forests (MoEF), Government of India.
(ii) Permission sought from Conservator of Forests of the region where exploration has to be carried out.
(iii) Field diary.
(iv) Collection bags/baskets.
(v) Paper bags (preferably made up of brown paper or news paper bags depending upon the availability).
(vi) Hand lens.
(vii) Cutting tools (Knife, small axe, hammer and chisel). The tools should be properly covered.
(viii) Pencil/pen.
(ix) Stapler/paper pins, waxed paper.
(x) Compass for navigational purpose.
(xi) Portable light source which is required in some shady forest localities where the fungus is sometimes required to be photographed. Some ultra light portable lights are available which can be carried easily.
(xii) Ethanol/Acetone in a small bottle (to be carried by the collector in the field). It is the personal experience of the author that in the dense forests, one is prone to be mosquito/wasp/honey bee bites. An application of these reagents on the site of attack act as an antidote and gets one relieved of the pain as well as the inflammation.
(xiii) Equipment required for setting temporary laboratory, if moving for few days far off from the laboratory. These basically include: Storage boxes, portable dryer, insecticide, emergency light, portable microscope to examine temporary slides and a good digital camera with macro-photographic facility.

3.3.2.2 Collection of the Specimens in the Field

While collecting the specimens in the specialized niches, care must be taken to leave some of the specimens in the collection locality. This helps in further dispersal and in the long run helps conserve the diversity. One should collect the specimens without damage and care should be taken that fructifications should not be torn apart. They should be collected intact along with a part of the substratum. Each

specimen should be placed in the paper bag along with the collection number, name of the host/substratum, locality on the label along with date of collection (pre attached to the paper bag with a stapler or gum). After putting the specimen in the paper bag, it should be folded on the top and sealed partially with a paper clip, so that the specimens do not fall in the basket or large bag during transportation to the temporary laboratory. Storage boxes with multiple chambers can be used to carry the delicate specimens. However in this case the collection number of the specimens along with the host/substratum should be noted on the labels to be put inside these chambers. The field data can be noted down in a diary. Plant diversity at the place of collection has to be noted down which is of great help while determining the inter-relationships if any at a later stage.

Do's and Do not's during the collection period:

1. Ask/employ locals in the vicinity of the forests if they know about these fungi. They can guide you in lesser time to specialised niches.
2. Devote more time in exploring a particular locality so as to explore it extensively than moving around in large areas. The resupinate fungi are most abundant on the underside of the fallen twigs/logs and broken branches still hanging from the living trees.

Do not's:

1. Do not use plastic bags for storage of the specimens in the field as well as later on because they encourage growth of moulds like *Penicillium*, *Aspergillus* etc. due to accumulation of moisture.
2. Do not put two taxa in one paper bag.
3. Do not put large number of fructifications in one open bag.
4. Do not risk yourself unnecessarily while collecting specimens from difficult niches.
5. Do not roll the large logs towards yourself while exploring fungi from the underside.
6. Do not put your uncovered hands in the cut/deep grooves of the tree trunks. You may be attacked by insects or snake.

3.4 Processing

3.4.1 Spore Print

Before drying the specimens, two specimens of the collection (if the specimens are small) or two pieces of collection (sufficiently moist) are selected and put on the black chart paper as well as glass slides (used for microscopy) with hymenial surface facing downwards. If the specimens are not moist, they are dipped in water for 30–60 min and then used for taking the spore print. The spore print is taken on both (black paper and slide) with the collection number of the specimen. The spore print

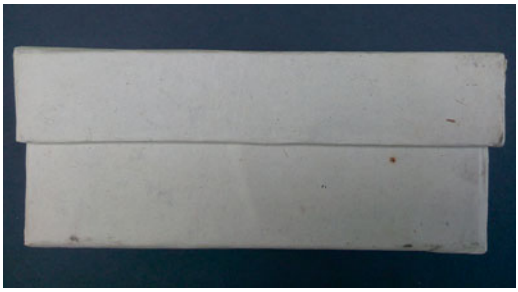

Fig. 3.3 Cardboard box

on the glass slide can also be used in culturing these fungi later on as well as in Scanning Electron Microscopic (SEM) studies of the spores. Both of these are covered with cellophane paper to avoid dust and contaminating spores of anamorphic fungi. The spore prints are checked after 10–12 h and packed. The slides with spore prints are covered with cellophane paper tied on both the ends with rubber bands whereas the paper with spore prints is folded four times and closed with adhesive tape. The paper used is of 4×4 inch which when folded makes to 1 inch square. These can be packed in a cardboard box Fig. 3.3.

3.4.2 Field Notes/Characters

A field performa can be used while collecting these fungi. The following points are important and are to be kept in mind while collecting in the field:

Most of the morphological features of the specimens are noted down while fresh and before drying.

The size of the specimen is noted as soon as possible because it changes to a larger extent in case of specimen with high moisture content upon drying. However, the fungi with perennial fructification change little. Similarly colour is compared with a standard colour chart. In our laboratory as also in the field laboratory, the colour of the specimens is compared with colour chart by Kornerup and Wanscher (1978). The other important morphological features to be noted down are the shape of the fructification, the surface of the pileus (if present) and the pore surface. Since most of the good digital cameras with fixed zoom lens have a macro-mode, one can photograph the pileus as well as pore-surface from close distance of the fresh specimen. The colour photographs of the specimens are taken before drying.

The specimens are described for macroscopic characters for identification as soon as possible, since in most of these fungi, particularly with effused fructifications, the growth of vegetative hyphae starts from the fructification after few hours of storage in the temporary laboratory which obstruct the hymenial layer. They should be examined as early as possible.

3.4.3 Culturing of the Fungi in the Field

It is better to culture the fungi *in vitro* on the PDA (Potato dextrose Agar) or MEA (Malt extract Agar) slants or petri plates in the temporary laboratory set at the main station within 24 h of the collection. The fungi are cultured from the germinating spores or from the tissue of the context. The cultures from the basidiospores are obtained by obtaining a spore print on the sterilized slide and then transferring these to the sterilized medium aseptically. A part of the inner tissue of the context is transferred with the help of a sterile needle to the sterile basal medium in petri plates or test tubes to obtain cultures. It is the personal experience of the author that these fungi are relatively easy to isolate *in vitro* when fresh than after few days of the collection. The fungi which could not be cultured in this way can be isolated from the spore prints taken on the slides. These can be isolated through single spore isolation techniques. The details regarding the various media and the isolation techniques are discussed in the section on *in vitro* isolation and preservation.

3.4.4 Preservation of the Specimens

The specimens are preserved by drying in the hot air oven. These are dried in an improvised wooden folding box like structure with three chambers. The base of the lower most chamber is fitted with hot air blower. The upper chambers are separated from the lower chamber by a horizontal wire gauge trays through which the hot air can pass. The upper most chamber is fitted with sliding cover divided into four sections. These can be opened or closed depending upon the specimens to be dried. The hot air after drying the specimens passes through the outlets in the upper chamber. The temperature in the upper chamber is maintained between 35 and 45 °C by adjusting the thermostat of the blower from position indicated on it. The blower is also replaced in some conditions by one or two electric bulbs of 60 W/100 W. The specimens retain their original colour in this method. The specimen put in the paper packet bags with labels can be placed as such in the dryer. This also rules out the mixing of specimens. After drying, these specimens are shifted to paper envelops of the size of 15×12 cm made of white computer stationary. Large number of genera of these fungi like species of *Ganoderma*, *Trametes* and other are infested with insects which make a powdery mass of fructification as a whole or a part of it. Commercially available insecticide like 1–4-Dichlorobenzene or Naphthalene balls are placed in each packet. These packets are than arranged in card board boxes of the size of $40 \times 17 \times 13$ cm in a vertical position. In order to protect the collections from insect attack, insecticidal balls are also put in these boxes. Large specimens and those belonging to genera like *Trametes* which are most susceptible to insects are further placed in zip lock polyethylene bags. Before placement of these specimen packets, it is ensured that the fungi does not contain traces of moisture. In order

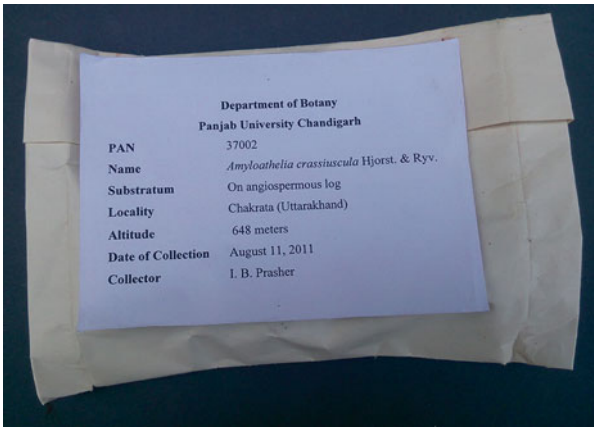

Fig. 3.4 Paper packet with label

to ensure this, the packets are placed for 1–2 h in the electric hot air oven at 45–50 °C. Each specimen paper packet is labelled with relevant data depicted in Fig. 3.4 which include: Name of the specimen, locality, substratum, Name of the determinator and Herbarium number.

3.4.5 Deposition of the Specimen

The specimens are deposited and arranged, in the herbarium after identification. One of the best ways to arrange the specimens is to arrange them alphabetically according to family, genera and species. The collections pertaining to species are arranged alphabetically with in genera. These are placed in card board boxes labelled with the name of the genera. The boxes pertaining to individual genera are arranged in the same fashion in a steel cupboard/almirah according to the family. A large family may be assigned to a single almirah/cupboard. The left out space may be utilized for the later on deposition of collections. The placement data is uploaded/stored in a computer in the herbarium which makes the retrieval easy and comfortable. The type specimens are normally stored separately for restricted access by the users only with the approval of the Curator of the herbarium.

3.5 *In Vitro* Isolation and Preservation

In order to study the decay characteristics and to study the physiological and biochemical parameters of these fungi; to be employed later on for biotechnological applications; pure and auxenic cultures are to be obtained. These are obtained by either single spore isolation technique or single hyphal tip isolation method. A brief

procedure adopted for the single spore isolation (based on Choi et al. 1999) is briefly described below. A piece of fructification with hymenophore is cut from the fructification and is placed on the inside of the Petri dish using Vaseline. Alternatively it can be fixed with the help of double adhesive tape. The Petri plate is then sealed with parafilm and left horizontally over night at 25 °C. In case of fertile specimens, the basidiospores fall on the agar surface.

3.5.1 Single Spore Isolation

In this method either a part of the spore print (taken in the field) or the hymenial layer of the specimens is used for making a spore suspension in sterilized distilled water. This suspension is then poured in pre-sterilized petri-plates containing sterilized 2 % agar-agar medium (2 g agar-agar in 100 ml of water) with the help of a pipette. The number of spores/ml of the water is pre-determined with the help of Haemocytometer. The petri-plates with spore suspension are placed in upright position for 4–12 h depending upon the ability of the spores to germinate which can be determined before pouring the suspension. The petri-plates are inverted to decant the water after the pre-determined period required for germination of spores. The germinating spores are marked in black with fine marker pen on the inverted side of the petri-plates under low power (4×objective) of the microscope. These are then lifted along with the agar with the help of a biscuit cutter needle and transferred to petri-plates or test tubes containing semi-synthetic sterilized media and incubated at optimum temperature.

An alternative method of obtaining the single spore cultures is to allow the spores to germinate and form a colony on the inoculated agar plates. When the individual colonies are about 2–5 mm in diameter, they are transferred to petri-plates or tubes containing the medium. The optimum temperature for most of these fungi has been found to be between 24 and 28 °C. It has been observed that suspension made from spore prints obtained in the field give better results (if they are properly covered) than spores obtained from the specimens collected few days before isolation.

3.5.2 Single Hyphal Tip Isolation

The cultures obtained from the hymenial tissue in the field contribute significantly to this procedure than the isolations from the specimens which have been collected few days earlier from their culturing. The success rate of the culturing the dried specimens is often not more than 40 %. The single hyphal tips can be marked on the inverted side of the petri-plates supporting cultures under low magnification of the microscope. These are than cut with biscuit cutter needle and transferred to petri-plates or test tubes containing synthetic/semi-synthetic media.

3.5.3 Composition of Different Media

The most commonly used media for isolation and maintenance of culture of these fungi are as follows along with their composition.

3.5.3.1 Potato Dextrose Agar Medium (PDA)

Composition	g/l
Potato (scrubbed and diced)	200.0 g
Agar	20.0 g
Dextrose	20.0 g
Distilled water	to make 1.0 L

Boil diced potatoes in 500 ml of distilled water, filter through cheese cloth, add distilled water to the filtrate to make 1.0 L. Add agar to filtrate and dissolve, then add dextrose.

Alternatively Potato Dextrose Agar medium concentrate manufactured by HIMEDIA, can be used. In this 39.0 g of the concentrate is to be mixed in 1.0 L of distilled water to get desired composition.

3.5.3.2 Yeast Extract Peptone Agar Medium (YEPA Medium)

Composition	g/l
Agar	15.0
Peptone	5.0
Yeast extract	3.0

Alternatively Dissolve 23.0 g powder concentrate to distilled water to make 1.0 L. The above medium is manufactured by Sisco Research Laboratories.

3.5.3.3 Malt Extract Agar Medium (MEA Medium)

Composition	g/l
Agar	18.0 g
Malt extract	20.0 g
Distilled water	to make 1.0 L

All the isolation/basal media mentioned above are sterilized at 16 lbs psi steam pressure at 121 °C for 30 min in an autoclave.

3.5.4 In Vitro *Preservation*

The cultures of these fungi are preserved in various ways. Some of the method employed include:

3.5.4.1 Storage of the Stock Culture

Storage of the stock culture in screw capped 1 inch diameter test tubes at ±4 °C in a frost free refrigerator. These cultures can be revived after 4 months.

3.5.4.2 Preservation of the Culture in Mineral Oil

Liquid paraffin, sterilized at 15 lbs psi for 2 h and cooled is added to the agar slants on which the fungal cultures are growing. Whole of the agar and fungal culture should be submerged in the oil. The tubes are kept in an upright position at a temperature of 15–25 °C. The level of the oil in the test tubes is checked at regular intervals. To revive the culture, a part of the culture submerged in mineral oil is removed and placed on the culture medium after draining the oil.

3.5.4.3 Immersion in Distilled Water

This is a low-maintenance and cost-effective method to preserve these fungi. The screw capped test tubes with cultures on the agar slants are completely submerged in sterilized distilled water in an upright position. Alternatively, the procedure involves cutting of the agar plugs from the edges of actively growing cultures and placing them in sterilized distilled water in screw capped tubes. It has been found that the fungal culture of these fungi survive for more than 2 years at 5 °C, in our laboratory which is in conformity with the findings of Marx and Daniel (1976) and Richter and Bruhn (1989). In our laboratory we subculture these isolates after 12–18 months. In order to save space, these culture plugs can also be stored in small, sterile, screw-cap cryovials and submerged in distilled water.

3.5.4.4 Freezing (After Ito 1991)

Discs are cut from the actively growing culture of these fungi on the petri-plates. These are aseptically placed in sterile cryotubes containing 10 % glycerol in distilled water. The cultures are preserved for up to 60 months at −80 °C.

3.5.4.5 Lyophilization (After Croan 2000)

This method is mostly applied to fungi which form large number of small propagules. The fungi cultured in our laboratory have not been preserved by this method.

3.6 Examination

The examination of fresh as well as dried specimens is accomplished by using the following mountants and stains to observe sterile and fertile elements microscopically. The microscopic details are combined with macromorphological features to describe a specimen for establishing an identity. For determining the identity of the specimen in the laboratory first of all macromorphological features are taken into consideration. These include: the size and the form of fructification; the pileus, its form, surface characteristics, shape and size of the pores; consistency/thickness of the context and tubes. The microscopic/micromorphological features are noted after sectioning the specimen along with the tubes, if present. These include: shape, size and dimensions of the hyphae, basidia, spores and other sterile elements. Sections from various zones of the fructification are observed for accurate and reliable conclusion. All the measurements concerning the elements of the fructification represent a mean of 30 reading concerning that element. Spores and hyphae are termed as amyloid if they turn blue or grey and dextrinoid if reddish brown. The following mounts and stains are used:

(a) 3 % KOH solution in water to record the colour and characters of the various structures *viz*.: hyphae, cystidia, basidia, basidiospores and setae etc.
(b) Cotton blue in lactic acid: it rapidly stains the cytoplasm of fungal cells. It is prepared by dissolving 0.01 g of cotton blue in 100 ml of 85 % of lactic acid while heating gently in a glass beaker. The cool solution is filtered and stored in a dark coloured bottle. For staining the hyphae, thin sections of the tissues of the context are revived in 3 % KOH solution, washed with excess of water and placed in cotton blue in lactic acid. The tissue is slightly warmed over a spirit lamp. This stain determines the cyanophyllous/acyanophyllous nature of the spores and other sterile structures depending upon whether the wall is stained blue or not.

Melzer's reagent: It is used to note the amyloidity or dextrinoidy of the various regions. Melzer's reagent is prepared as follows after Singer (1962): Iodine 0.5 g, Potassium iodide 1.5 g, Chloral hydrate 22.0 g and distilled water 20.0 ml.

Eosine and Phloxin: 1 g Eosine or Phloxin in 100 ml of water.

The sulfovanillin [distilled water 2.0 ml, Sulphuric acid (pure) 4.0 ml, Vanillin 0.5 g] is used for staining gloeocystidia. A fragment of dried/fresh specimen is put in the stain for 2–3 min and after crushing, it is observed. The structures stain black in the positive reaction.

All the measurements are taken in 3 % KOH solution. The diagrams have been made from a single collection. The structural details concerning hyphae, cystidia, basidia, basidiospores, basidiole and other sterile accessory structures have been drawn with the help of Olympus drawing attachment. The diagram of hyphae, basidia, cystidia and basidiospores have been drawn at a magnification of 2,000(×2,000) where as larger structures at ×400 or ×800 magnification. All the line diagrams have been provided scale. All the scales are equal to 10 µm.

The photographs have been provided only for specimens which have been photographed in the field or those whose morphology colour has not changed much after drying. No photographs have been given for the specimens studied from the herbarium. The following information is being provided for each genus in the order stated:

1. Name
2. Brief description
3. Reference to the legitimate report
4. Number of species reported in the world (after Kirk et al. 2008, Mycobank and Index fungorum)
5. Literature
6. Type species
7. Number of species being reported from the Himalayas.

The following information is being provided for individual species in the order stated:

1. Name of the species with reference to the published report. The synonyms/basionyms are given only for those species which have been recorded earlier under that name from Himalayas/India.
2. Description: Both macro and microscopic details. The description of each species is supplemented by the line drawings where ever possible coloured photograph of the specimen studied has been provided. The data concerning the frequency of occurrence (rare, common, abundant) along with the life form i.e. saprophytic/parasitic is also given.
3. Distribution: It includes the abbreviated name of the States (HP: Himachal Pradesh, UK: Uttarakhand, J & K: Jammu & Kashmir, N: Nepal, B: Bhutan, M: Manipur, Mi: Mizoram, WB: West Bengal, AP: Arunachal Pradesh, As: Assam, T: Tripura, Me: Meghalaya) along with stations.
4. Collection examined with herbarium number for the specimen studied along with abbreviated name of the collector (G. S. Rawla = RW/GSR, S. S. Rattan = R/SSR, R. S. Dhanda = D, M. P. Sharma = MPS, H. S. Khara = K/HSK, G. S. Dhingra = GSD, S. S. Virdi = SSV, I. B. Prasher = IBP and Lalita = L) Some of the collections have been deposited without the name of the collector. They are cited as such in the text. The collections examined (mentioned in this work) are those which have been worked out for making morphological, anatomical details. The other collections deposited in the herbarium are not mentioned.
5. Substratum.
6. Remarks

The photographs and line drawings provided in the monograph are the property/ copy righted materials of the different agencies of the Government of India (U.G.C, CSIR (HRDG), MoEF) which is the outcome of the research projects undertaken by the author during the last 10 years. The author holds the right to publish this data; under the guidelines provided by different agencies; and is required to submit the published data to the agencies concerned.

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