IV. MATERIALS AND METHODS

The areas of collection were restricted to some districts of West Bengal viz. Calcutta, Bankura, Midnapore, Howrah and Darjeeling. Accordingly, frequent tours were made in those areas for the purpose of collection and field study. The different areas as attended are presented by symbols in relevant maps (Text fig. 1). During the collection, the habit and habitat and other necessary field datas (e.g., soil condition, mycorrhizal or non-mycorrhizal, locality), the morphological characters, especially the colours (if any) of the specimens in natural conditions) were noted along with the association as and when possible. The collected specimens were brought into the laboratory as soon as possible. Then the critical observations (macroscopic, microscopic, and chemical tests) of each specimens were done.

The macroscopic observations included the study of stipe, pileus, lamellae, spore print etc. The microscopic observations included the study of hyphal analysis of the context, hymenial layer, pilear and tramal layers etc.

For microscopic study a small piece of tissue from upper surface of the pileus, from pilear trama, hymenial trama and stipe surfaces were placed on a glass slide containing a few drops of 10% KOH solution and after few seconds it was covered with a coverglass. The coverglass was gently tapped to spread out the tissues. After this, the types of tissues present in the pileus,

stipe and hymenial trama and basidia, cystidia and gloeocystidia etc. were observed under the microscope. Free hand sections of the fresh specimens were also made and observed similarly under the microscope.

The mass of spores collected from the spore print was placed in a clean dry slide containing a few drops of Melzer's Iodine and after few seconds the coverglass was placed on it and observed under the microscope. The types of reactions i.e., whether amyloid (if positive, spore wall colour will be bluish tinged) or pseudoamyloid (i.e., spore wall colour not so prominently bluish tinged) or dextrinoid (i.e., spore wall colour changes other than blue) were noted.

Necessary drawings and measurements were also made. The microscope was standardised by placing the stage micrometer and ocular micrometer as usual. The Q value (i.e., length/width ratio) of the spores including apiculus were taken from the average mean value of the 20 spores selected randomly from the spore slides.

Along with this, free hand vertical sections of different parts of the basidiocarps were also made to observe the internal structures in details. The gross internal structures are presented by camera Lucida drawing.

Spore print preparation

The pileus of the freshly collected specimen was cut and placed in a clean dry sheet (1/2 part white and 1/2 part black) by upside down to the sheet for spore print preparation. The colour of the spore mass were noted from the colour identification chart (Flora of British Fungi) - Published by Her Majesty's Stationery Office, Edinburgh, 1969.

All the collected specimens were dried and kept in the herbarium of Mushroom Research Centre, Mycology (Basic and Applied) Laboratory, Department of Botany, Calcutta University, Calcutta-700 019, India.

The following chemical tests were done on the fresh specimens and also on the thin free hand sections of the fresh specimens.

The following reagents and dyes were prepared and used.

(I) Melzer's Reagent

Chloral hydrate	:	100.0 gm.
Potassium Iodide	:	5.0 gm.
Iodine	:	1.5 gm.
Distilled water	:	1000 c.c.

This reagent was mainly used to study the nature of the inner walls of the light coloured spores and in the hyaline or

light coloured structures. The reaction depending upon the final colouration of the preparation, wall is called amyloid (or pseudo-amyloid) if positive, and inamyloid when negative. The amyloid reaction is nearly blue-black, pseudoamyloid is much faint blue-black and dextrinoid reaction is yellow to brown and inamyloid reaction having no reaction i.e., original colour of the spore remains as such.

(II) Potassium hydroxide (KOH) 10% in distilled water

This chemical was applied on the pilear cuticle surface. If the reaction is positive, it darkens certain layers of the pilear cuticle as found in Gymnopilus. In Crinipellis mirabilis, the epicuticular hair becomes grey or green in KOH (Singer, 1942).

(III) Concentrated sulphuric acid (H_2SO_4)

The action is instantaneous or almost so. The spore colour of certain Coprinaceae changes from black to pale-blue grey, while in other species there is no reaction and the black pigment is resistant. A drop of pure acid if applied to the gills of Amanita phalloides, gives a pinkish-lilac colour.

(IV) Ammonia (NH_4OH) 50% in distilled water

The colour of cystidia in Pholiota, Stropharia, Naematoloma turns a bright yellow. The trama of Xeromphalina caulicinalis and other closely related species turn red.

(V) Sulpho-vanillin

Chemically pure vanillin	:	0.5 gm.
Distilled water	:	200 c.c.
Pure sulphuric acid	:	4.0 gm.

The resulting solution is of a deep rich yellow and should be filtered through glass wool and handled very carefully and must be used on fresh material. The reagent is commonly used for Russula species. A few drops on the stipe of R. rosea turns it a deep red colour.

The macrocystidia, dermato-pseudo-cystidia, some oleiferous and laticiferous vessels of species of Russulaceae turns blue and cystidia, gleocystidia basidia, ciliate dermatosystidia and the primordial hyphae of the members of Russulaceae turns hyaline to rose colour within five minutes of applying the reagent on the thin piece of cap cuticle, or gill tissue or stipe tissue.

(VI) Guaic (ordinary Guaiac tincture)

The oxidases if present in fungi react with Guaiaconic Acid. A blue (or green) to purple spot on the surface of the stipe indicates a positive reaction. In Agarics and Boletes it reacts positively (Sing 1975).

(VII) Guiacol (water solution, slightly below the saturation point)

Reaction, if positive, from Salmon-colour-orange to rose colour or bluish pink, slowly darkening to dark copper or chocolate colour in most cases, the base of the stipe is always most sensitive; the reaction is useful in the Russulaceae, Tricholomataceae, Amanitaceae (Sing 1975).

(VIII) Phenol (Carbolic acid) : 2% in distilled water

Used with Russulaceae, Amanitaceae and Tricholomataceae; a chocolate or deep purplish violet colour indicates a positive reaction. If, after 20 minutes, there is no distinct change, the reaction is regarded as negative.

(IX) Ferrous sulphate ($FeSO_4$) : 10% in distilled water

Used with Russula, Tricholoma, Tricholomopsis and many Boletaceae. The colour reactions are of several categories :

1. Negative reaction - no colour change;
2. Some shades of olive, green, blue-green or blackish-green colour of the context or surface of the stipe;
3. All shades from pure pink to grey;
4. Blue or green-blue to slate grey;
5. A variable colour effect on the cuticle of the pileus.

(X) Henry's reagent

Thallium oxide	:	2 gm
Concentrated Nitric Acid (HNO_3)	:	1 cm^3
Concentrated hydrochloric acid (HCl)		4 cm^3
Sodium bicarbonate ($NaHCO_3$)	:	1 gm
Distilled water	:	10 cm^3

This is a poisonous reagent and useful for identification of Agaricus xanthodermus from other edible species; a few drops applied to the cuticle of the cap gives a brick-red positive reaction, on A. xanthoderma there is no such reaction.

(XI) Schaeffer's cross reaction : (Aniline + HNO_3)

Very useful reaction for the confirmation of the members of Agaricaceae. A streak of conc. HNO_3 is made on the pileus of Agaricus specimens, then a crosswise streak is made with Aniline oil. The positive reaction is an orange-red to fire red discolouration.

(XII) Aniline (aniline oil and aniline water)

Pure aniline oil, or 50 percent in distilled water. It gives a red to copper-red colouration on the context of the stipe and causes a central stained sport, surrounded by a grey or brightly coloured zone, on the gills of Russula, Boletus and Agaricus species and for several genera in the Aphyllophorales.

(XIII) Phenol-aniline : 2% in distilled water

A few drops of aniline oil with phenol in water. This reagent must be applied on dried materials. The reaction is from nil to nearly black after prolonged exposure.

(XIV) Alpha-naphthol

The reagent need not be accurately mixed; a few crystals are dissolved in about 2 cm^3 of 90 percent alcohol and then about 4 cm^3 of water are added. The solution reacts rapidly with the context of the stipe of Marasmius grandisetulosus causing a deep wine colouration.

(XV) Pyrogallol

5 percent in distilled water is said to give richly coloured reactions with the context of Russula, Marasmius and other mushrooms.

(XVI) Pyramidon (saturated soln. in distilled water)

A positive reaction indicates a light lilac colour on the context of the stipe; used in Tricholoma and Russula.

(XVII) Formaldehyde 40% in distilled water

A positive reaction indicated by a change in the colour of the context. It is a slow reaction, 20 minutes being required to accomplish the change; used in Tricholoma, Russula and Boletus.

(XVIII) Aceto-carmine (for Carminophilous basidia)

Test must be made on dried material. 2-3 drops of the reagent poured on a small piece of gill on a glass slide, and then heated gently without allowing the material to dry out. Finally mounted in a fresh aceto-carmine, then covered by a coverglass and tapped gently to splay out the material and then examined under an oil immersion lens. Basidia with black staining granules indicate a positive reaction.