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# KARYOLOGICAL STUDIES ON VEGETATIVE MYCELIUM OF *LENTINUS SQUARRULOSUS*: A FUNGAL SPECIES FROM THE COOCH BHEAR DISTRICT (DOOARS REGION) OF WEST BENGAL, INDIA.

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***Abstract:***
*The cytological investigations and analysis of different members of the fungal kingdom, till today, represents an unexplored area in the field of mycological research. Owing to its miniscule contour and very short period of divisional phases in addition to the partial penetration of different stains (used in conventional light microscopy) as a result of retention of nuclear envelope during nuclear division, the fungal chromosomes remained as not so very popular subject of cytogenetic research. Over the years numerous attempts have been made to elucidate the finer details of the nuclear as well as its divisional stages inside the vegetative filaments mainly from the meiospores instead of mitotic stages. Analysis of the ultrastructure of chromosome and chromatin materials from Basidiomycetean fungal members has not only been fascinating because of its novelty but also can highlight greatly on the phenomenon of chromatin compaction into condensed chromosomes during nuclear division and migration in the vegetative filaments. Several unique features after orcein staining were observed which mainly composed of resting nuclei in the vegetative mycelium followed by typical anaphasic division of the vegetative nucleus enclosed within nuclear membrane. Lentinus cytology also revealed mesh-like chromatin material with nucleus enclosed within intact nuclear membrane. It also possessed late anaphasic nuclear division with retention of intact nuclear membrane with a trailing nucleus. Developing basidium had two resting nuclei with clear distinction.*
***Key words:*** *light microscopy, white macrofungi, Basidiomycetes, chromatin compaction, trailing nucleus and asynchronus division, resting nucleus in vegetative mycelium,*

## Introduction:

The state of West Bengal (88,752 sq. Km), India, extends from the Himalyas in the north to the Bay of Bengal in the south and owing to this varying elevation range, it supports rich biodiversity. Darjeeling Hills forms a part of the Eastern Himalyas and icnlueds Sandakfu, the

higher peak of the state. The narrow Terai and Dooars Region separates this region from the plains, which in turn transitions into the Ganges Delta toward the South. The Terai and Dooars region consititues the plains of Darjeeling, Whole Jalpaiguri District, and the upper region of the Cooch Behar in West Bengal. The entire region is made up of sand, gravel and pebbles laid down by the Himalyan rivers like Teesta, Torsa, Raidak, Jaldhaka, Sankosh and several other small river lets.

The Terai and Duars region of West Bengal (Jalpaiguri and Cooch Behar District) is rich in different floral and faunal biodiversity. Different unreported species of microbes, fungi and plants have been dwelling this vast area which needs immediate attention from the scientific community. Owing to these persisting favouring condition this Terai region has also been found out to be a "Biodiversity Rich" area with innumerable unknown, unidentified and unexplored (at cytological level) fungal species. This present investigation is an scientific attempt which has been initiated keeping the goal ahead to elucidate the cytological peculiarities (especially the karyological features) in the vegetative mycelium of the collected species (*Lentinus squarrulosus)* immediately after proper fixation and staining.

Fungal chromosomes are too small and very difficult to observe under compound microscope; hence there are few reports available of successful examination of them compared with reports on plant and animal chromosomes. Furthermore, chromosomes usually differ little in size, so it is difficult to distinguish between them to prepare for karyotyping. In filamentous fungi also, mitotic chromosomes have rarely been studied. Such studies have been hampered by several obstacles such as the small size of the mitotic chromosomes in comparison with meiotic chromosomes, the asynchronous movement of chromatids during nuclear division and the lack of proper techniques for the preparation of chromosome spreads. Consequently, information concerning the morphological nature of chromosomes in filamentous fungi is heavily biased toward meiotic chromosomes, in marked contrast to the situation in higher plants and animals (Taga and Murata, 1994). Although mitotic chromosomes of filamentous fungi are more difficult to observer than the meiotic chromosomes, mitotic observations are necessary in some cases. For example, sexual stages are when unknown or difficult to form in laboratory in a considerable number of species including many important plant pathogenic fungi.

Not many fungal species have been examined cytogenetically to date. They are mainly Ascomycotina. There is shortage of data concerning Basidiomycotina (especially wild

agaricales) and Zygomycotina. Some of the authors have stated that it has been generally easier to observe meiotic chromosomes than those from mitosis. Mitotic chromosomes are usually smaller in comparison to the meiotic ones. Furthermore, usually a well-defined "metaphase plate" remains absent in addition to the disjunction of sister chromatids, which is asynchronous (Aist and Morris, 1999). Here mitotic observations are necessary in some cases, for example, when a given fungus does not undergo sexual processes or does not perform them in laboratory conditions. The use of two different methods examining the same species: conventional light microscopy and GTBM (germ tube burst method) reveals that results might not coincide. In some cases counts of chromosomes in asci, by conventional light microscopy, leads to underestimation of chromosome number comparing with data of PFGE (Pulse field Gel Electrophoresis) or GTBM; for example in *N. Haematococca* (Taga *et al.,* 1998) . This suggests that re-examination of data from past cytological studies is needed.

Cytogenetic studies of chromosomes during mitosis or meiosis leads to the clarification of the number of chromosomes and their behavior during cell division. They also reveal peculiarities of chromosome structure. Knowing these features, adding data from other molecular tools, gives a full and complex overview of any fungus being studied, at three conceptual levels viz: genome, chromosome and DNA.

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Our present work aims to focus on reporting the different cytological features coupled with orcein staining of the vegetative mycelium of the collected Basidiomycetean fungi (*Lentinus squarrulosus*) growing in this phytogeographic region to enrich the present knowledge base of cytogenetic/cytological characteristics of this very species.

**Materials and Methods**

Immediately after collection the fungal specimens form fields are brought to the laboratory, fixed in New Comer's solution ( Isopropyl alchol: Propionic acid:Petroleum ether: Acetone= 6:2:1:1) and preserved. Within 7 days of preservation the specimens were subjected to orcein staining.

**Preparation of the stain:**

About 2 g Orcein powder was weighed and brought to simmering 45% acetic acid solution 100 ml and boiled. The mixture was kept at simmering condition for about 15 minutes and gently stirred. Finally the solution was cooled to room temperature and filtered with Whatmann No-1 filter paper and kept in glass stoppered bottle and kept at room temperature for further use. The fungal tissues were cut into small pieces, washed several times in distilled water and then hydrolyzed in 0.17 M NaCl for 20 minutes at room temperature. After 20 minutes the hydrolyzed fungal tissues were washed 3-4 times in distilled water and then fixed in acetic alcohol (1:3) mixture for 10-15 minutes at room temperature. After keeping for 15 minutes at room temperature fixed tissues were rinsed with distilled water several times to wipe out the last trace of the mixture. The tissues were then subjected to acid hydrolysis with 1(N) HCL at 60˚C for 10 minutes following intermittent shaking. After 10 minutes the hydrolyzed tissues were rinsed several times in distilled water to remove the last traces of acid. Finally the tissues were subjected 45% acetic acid rinsing for 5 minutes before staining. After 5 minutes, the tissues were kept in clean test tubes, mixed with 2% orcein solution and kept at 60˚C for 10 minutes, immediately cooled and again preserved in 45% acetic acid solution and observed for cytology.

**Results: *Lentinus squarrosulus* Mont.**

**Description of the specimen:** Pileus 2–8 cm wide, sub-infundibuliform, with a deep depression in the centre, white to cream coloured, turning some what brownish colour in age, coriaceous and flexible when fresh, becoming stiff smooth on drying, concentric zone of small squamules present, ranging from subrevolute to entirely appressed, margin downcurved, initially involute, thin, glabrous, sometimes with minute scales. Gills crowded, concolorous, deeply decurrent, white to cream coloured when young, brownish with age, edge serrate, lamellulae of four lengths. Stipe central, sometime ecentric, cylindrical, 3.5 cm long, whitish at first, brown in colour at maturity, smooth with minute squamules, solid, tapering below. Ring and volva absent. Context near about 2 mm thick.

Basidiospores 4.2–6.8 × 3–3.4 µm, $Q_{av}$=1.71, number of spores examined=30, oblong-elliptical, white, hyaline, smooth, thin walled. Basidia 13.6–18.7 × 3.4–4.2 µm, clavate, tetrasterigmatic. Lamella edge sterile. Cheilocystidia 11.5–28 × 3.9–5 µm, cylindrico-clavate, hyaline, thin walled. Generative hyphae 3.94-5.91 µm in diameter, hyaline, 0.39 µm thick, clamp-connexions present. Skeletoligative hyphae 2.76-7.88 µm in diameter, wall 1.97 µm thick, hyaline.

Hymenophoral trama irregular, loosely woven, hyaline. Pilipellis a loose epicutis, composed of 3.94–7.09 µm in diameter, hyaline hyphal cells, wall 1.18 µm thick. Stipitipellis composed of 7.09–7.88 µm broad, hyaline hyphae, wall 1.18 µm thick. Oleiferous cells absent.

Habitat: Frequent with dense population (in terms of number of fruitbodies) on the exposed moist wooden logs.

Edibility: Edible.

Remarks: The white macrofungi, *L. squarrosulus* superficially resemble *L. tigrinus*, but white, semi-erect squamules on both the pileal and stipe surfaces and also the crowded lamellae without denticulate edge differs this fungi from *L. tigrinus*. The same kind of distribution pattern resembles *L. squarrosulus* with the fungi *L. sajor-caju*.

**Discussion:**

In nuclear studies of fungi, the location of nuclei in cells, number of nuclei per cell, size of nuclei, and their behavior during different phases of growth and development are important. To study these details it is necessary to develop techniques to stain nuclei with maximum clarity, rapidity and simplicity.

Various nuclear stains are available, but only a limited number of them are useful in elucidation of fungal nuclear staining (Mogford, 1979). The available stains and techniques often do not give the necessary results for nuclear studies in fungi and also are useful only in particular isolates, species, or groups of fungi (Kangatharalingam and Fergusonm, 1984). This may be due to the fact that fungal nuclei have a very poor affinity for most ordinary stains (Mogford, 1979) . The wall materials also may have varying degree of affinity to these stains, thus interfering with the clarity of the stained nuclei.

From exhaustive literature search we have come to the conclusion that although different staining protocols have been attempted in elucidation of the karyological characteristics of different fungal species, till date a very few species have been subjected to proper experimentation. Different hardships have been encountered while these studies employed in staining fungal cell. The ideal solution would be to combine cytological studies (with simple staining, DAPI and Giemsa staining) and PFGE in karyotyping fungi, as in the cases of *N. crassa* (Orbach *et al.,* 1998), *E. Graminis* (Borbye *et al.,* 1992) , *N. haemotococca* (Taga *et al.,* 1998) ,

and *C. heterostrophus* (Tsuchiya and Taga, 2001) . Sometimes PFGE is not useful when chromosomes are large and similar in size, as in *Fusarium graminearum*. In this case cytological observation of four chromosomes is confirmed by a molecular method like genetic mapping (Gale *et al.*, 2005). The advantage of molecular tools, widely used nowadays, like amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP) or rDNA sequencing (Soll, 2000) is that they can be conducted on DNA without any (or with little) knowledge about the structure of fungal genomes.

In the present study the fungal fruitbodies of *L. squarrulosus* were fixed in New Comer's solution ( Isopropyl alchol: Propionic acid:Petroleum ether: Acetone= 6:2:1:1) which gave good results as after 7 days of fixation when the tissues were subjected to orcein staining gave good clarification of nuclear material. The fungal tissues were cut into small pieces, washed several times in distilled water and then hydrolyzed in 0.17 M NaCl for 20 minutes at room temperature (Singh, 1969). Sodium chloride treatment checks the undesired staining of the spore or hyphal cytoplasm. Ganesan and Swaminathan (1958) had reported that NaC1 removes RNA and certain basophilic materials from the cytoplasm which otherwise take up the nuclear stain and interfere with proper differentiation of the nucleus. Sodium chloride hydrolysis is not only simpler and quicker, but also gives better results as compared to HCl and certain other extraction methods tried herein. We also here employed the same recipe and obtained good results.

Another ascomycetean fungus *Erysiphe graminis* f. sp. *hordei* had been examined (Borbye *et al.*, 1992)[7]. In this case a protocol incorporated simple aceto-orcein staining which was found not sufficient to make the chromosomes of this fungus visible. Treatments including the use of colchicine 0.05% w/v, dimethylsulphoxide, hydrolamine hydrochloride and iron alum, prior to orcein staining, were found to improve the results. Seven to eight chromosomes could be well detected in cells at somatic metaphase establishing a lower number of n=7. In contrast to *T. aestivum*, chromosomes of *E. graminis* differed in size and shape. It was noticed that all metaphase cells were having a prominent nucleolus; thus the persistence of the nucleolus prevented a more exact assessment of the size of the nucleolus-forming chromosome. The other chromosomes could be assigned to at least three groups according to their size. The three largest chromosomes were triangular in size of 1.2×0.8 μm (approx). Two medium-sized chromosomes, approximately 0.8×0.3 μm, were rod-shaped. The two smallest chromosomes (0.4 μm) were circular. It was also observed that two of the largest chromosomes were often connected forming a butterfly-shaped body; they may be components of one very large chromosome. The results

obtained from field inversion gel electrophoresis seemed to confirm these findings (Borbye *et al.,* 1992).

Number of nuclei per cell is a very important character of vegetative mycelium in basidiomycetous fungi for the estimation of the taxonomic and evolutionary position of species. On the basis of nuclear characteristics groups of higher Basidiomycetes can be separated (Boidin, 1971). This author suggested that five primitive Basidiomycetes have had two nuclei, had clamp connections and were heterothallic. He regarded the multinuclear status of higher Basidiomycetes as a trend towards evolutionary advancement. Kuhner (1977) also considered morphological complexity in Basidiomycetes as connected with total or partial loss of clamp connections as evolutionary advanced character. Quantification of the number of nuclei in cells of the vegetative mycelium in 17 species of the genus *Agaricus* using the DAPI fluorescence staining method showed that it is different in definite species. The lowest number of nuclei per cell (2 nuclei) was found in A *bitorquis,* A *xanthodermus,* A *bresadolianus,*A *squamuliferus,* A *subfloccosus.* Some species contain between 2 and 4 nuclei per cell:A *abruptibulbus,* A *maskae,* A *excellens,* A *vaporarius,* A *sylvicolus,* A *macrocarpus* str.150. Cells of the remaining species contain from 4 to 6 and more nuclei per cell. All strains belonging to the same species show similar numbers of nuclei per cell (Wasser, 1980). An exeption is A *macrocarpus* where the two strains differ in this property. Due to the type of nuclear distribution in cells of the vegetative mycelium it would be possible to arrange the species investigated in an order showing an increasing evolutionary level, beginning with species containing 2 nuclei per cell and finishing with A *bernardiiformis,* containing 8-10 nuclei per cell. The criterion of number of nuclei per cell, however, is often contradictory to other evolutionary criteria such as shape of basidiospores, absence of clamp connections, level of ploidy etc (Wasser, 1985) A possible explanation for this could be the phenomenon of heterobatmy in the genus Agaricus (Wasser, 1980) .

Similar observations could also be found with *L. Squarrulosus* as observed in our study. The typical number of nuclei varied greatly in different locations within the same vegetative mycelium. In our study of *L. Squarrulosus,* we could encounter variable number of nuclei in the vegetative filaments (Fig 2L: Vegetative filament showing anaphase stages of nuclei followed by several fused nuclei) but the number of nuclei could be easily encountered in the growing apical tips of vegetative filaments (2n=2) and the nature of anapahsic movement of the dividing nuclei could be easily deciphered where the late anaphase division of vegetative nucleus showed intact

nuclear membrane, (Fig: 2I) with unequal division the nucleus showing a much smaller trailing nucleus (asynchronus division). Fig 2O-2P showed the typical perpendicular arrangement of resting nucleus in vegetative filament in groups of 3-4 which gives a clear count of nuclei (2n=6 and 2n=8). This peculiar arrangement of resting nuclei arranged in groups is atypical in this very genus.

*Lentinus edodes*, (Tanaka and Koga, 1972) had been studied for the cytological characteristics of the chromosome numbers and nuclear divisions at both mitosis in the secondary mycelia and meiosis in basidia. Matsuda (1976) had decisively reported a bead-like structure in the chromosomes of the resting nuclei in the secondary mycelia of this species by whole mount electron microscopy. Matsuda (1976) reported the differences of the shape of the nucleus and at least three types of nuclei were disting uishable: an oval shaped nucleus, an angular shaped nucleus and a rod shaped nucleus. Furthermore, the types of nuclei were disntinguishable due to differences of the chromatin viz, partially condensed nucleus and condensed nucleus. The partially condensed nucleus was to have some heavily stained condensed chromatic bodies which varied in shape, size and number. The condesned chromatic bodies were roundish, oval or in an irregular rod-like shape, and were varied in size. The number of chromatic bodies varied from one to eight in a nucleus and nuclei with two or three condensed chromatic bodies were most often observed (50%). The condensed nuclei were heavily stained due to the gathering of about eight variously sized condensed bodies. The darkly stained condesned chromatic bodies consisted of heterochromatin and lightly stained diffused chromatic regions were euchromatin (Heitz, 1928). The differentiation of heterochromatic and euchromatin is a very important cytogenetic parameter in the evolution of chromosome structure (Du Praw, 1970). The differentiatin of the chromosomes in *L. Edodes* may indicate the chromosomes of this species are similar to the chromosomes of higher organisms concerning this differentiation. In our studies of *L. Squrrulosus* we could see in Fig 2H: A developing cystidium showing mesh like fused nuclei enclosed within intact nuclear membrane showing highly stained nuclei (heterochromatic region) and lightly stained chromatin fibrils (Meshy appearance).

Clamp connections are characteristic features of many dikaryotic mycelia of Basidiomycetes (Buchalo *et al.,* 1983). It is widely accepted, however, that they are not common in all species of Agaricus. Singer (1961) reported the presence of clamp connections in the genus Agaricus but he did not mention definite species. Garibova & Shalashova (1973) during their investigations of mycelial morphology of Agaricus species found clamp connections only in A *campestris,* A

*subperonatus* (J. Lge) Sing, and paired clamp-like structures in A *bisporus.* Clamp connections were observed also in A *arvensis*, A *bernardii* Quel, apud Cke et Quel. (Wasser, 1985; Sonnenberg and Fritsche, 1989) and in A *comtulus* Fr. (Garibova, 1982). Clamp-like features were described in A *sylvaticus* (Wasser, 1985) . The majority of authors noted that clamp connections occured very rarely in vegetative mycelium of Agaricarales. Using light and SEM we investigated the clamps connections in A *arvensis* and A *campestris* which occured very rarely. In the present investigation of *L. squarrulosus* we could not observe any clamp connections.

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**Cytology of *Lentinus squarrulosus***

Fig 2A and 2B: Vegetative fruit body of *Lentinus squarrulosus*.

Fig 2C: Anaphasic division of vegetative nucleus encoled within nuclear membrane. X 1650.

Fig 2D: Apical tip of dividing vegetative filament showing numerous actively migrating nuclei. X 1650.

Fig 2E: Anaphasic division of vegetative nuclei away from the apical tip. X1650.

Fig 2F: Resting nucleus in the vegetative filament enclosed with intact nuclear membrane. X 1650.

Fig 2G: Resting nucleus showing dotted nuclear membrane and atypical configuration before getting into anaphase stage. X1650.

Fig 2H: Developing cystidium showing mesh like fused nucleus enclosed within intact nuclear membrane. X 1650.

Fig 2I: Late anaphase division of vegetative nucleus showing intact nuclear membrane. Fig showing unequal division the nucleus showing a much smaller trailing nucleus. X 940.

Fig 2J: Typical Anaphase division of vegetative nucleus inside intact nuclear membrane. X 1650.

Fig 2K: Developing basidium showing a spore. Enclosed within the spore can be seen the intact nuclear membrane showing two dotted resting nuclei. X 1650.

Fig 2L: Vegetative filament showing anaphase stages of nuclei followed by several fused nucleus. X 1650.

Fig 2M: Vegetative filament showing numerous nuclei arranged in groups.X 940.

Fig 2N: Tip-to-tip hyphal copulation showing fusion of vegetative nucleus in groups. X 940.

Fig 2O-2P: Typical arrangement of resting nucleus in vegetative filament in groups. X 1650.

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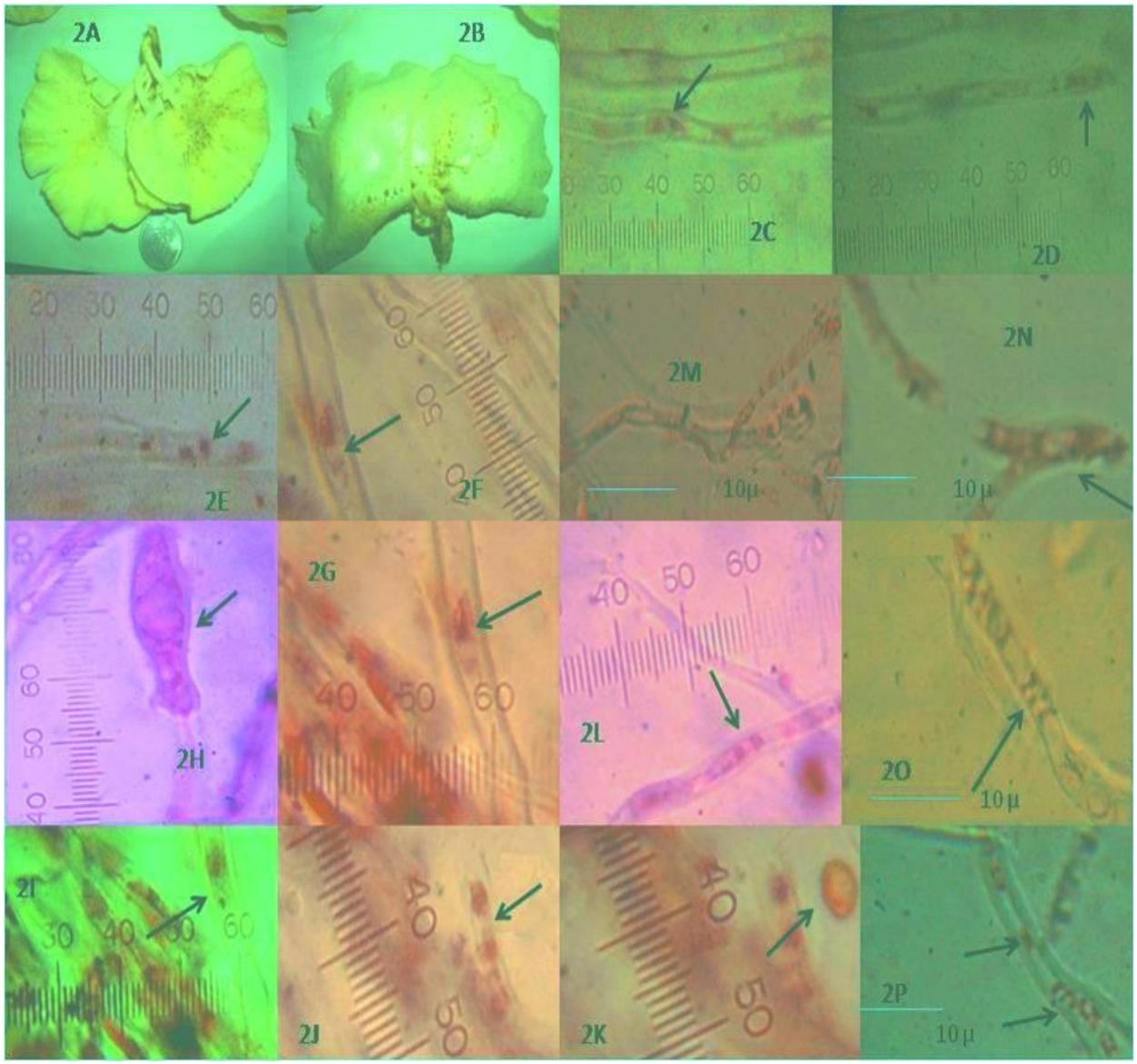


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