



## **Study on mycelium growth of *Calocybe indica* P&C (milky mushroom) on different substrates**

**Yin Yin Sint\*, Myat Myat Moe\*\***

### **Abstract**

*Calocybe indica* is one of the best edible mushrooms which can be grown at high temperature. Milky mushroom were collected from Hlegu Township, Yangon Division. Tissue culture were raised separately from different growth stages (stipe with small pileus, stipe with well differentiated pileus and mature fruiting body) were cultured on potato dextrose agar (PDA) and potato dextrose yeast agar (PDYA) media. After one week, it would start checking the growth of mycelium until within two weeks. Among them, stipe with well differentiated pileus of milky mushroom pieces tissues were gave the best of mycelium in PDYA media. The best growth of mycelium was conducted to find out the efficacy of different substrates such as rice grains and sorghum grains. Among the two different substrates, sorghum grains are the best for cultivation of milky mushroom. The bags were monitored for the growth of the mushrooms and yield in each of the bags were recorded.

### **Introduction**

Mushrooms are fleshy, spore-bearing fruiting body of fungi belonging to the subdivision of Basidiomycotina of the class Hymenomycetes. Mushrooms are amongst the most popular food items accepted the world over. Mushroom includes 14,000 to 22,000 species while the real number may be much higher associated with the underscription of species and the non-differentiation associated with overlapping morphological characters (Hawksworth, 2001). The mushroom defined as “a macro fungus with a distinctive fruiting body, large enough to be seen with the naked eye and to be picked up by hand”. Unlike green plants, mushrooms are heterotroph. Not having chlorophyll, they cannot generate nutrients by photosynthesis, but take nutrients from outer sources. Their body is composed of mycelium. Mycelium is the vegetative part of a fungus, consisting of a mass of thread-like hyphae. A mature hyphae forms fructifications most frequently protruding from the surface of the substratum.

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\* Lecturer, Dr, Botany Department, Dagon University

\*\*Professor ,Dr, Head of the Botany Department, Dagon University

Mushrooms fructifications are composed of two basic parts: the pileus and the stipe. Milky mushroom was first reported in India by Pukayastha and Chandra in 1974. It belongs to the kingdom fungi, phylum Basidiomycota, class Agaricomycetes, order Agaricales and family Lyophyllaceae. *Calocybe* genus consists of about 20 species of mushrooms, including *Calocybe indica*, which can be cultivated throughout the year in the entire of India even in hot humid climate (Kalha *et al.*,2011). It is becoming the third commercially grown mushroom in India after button and oyster mushrooms. (Purkayastha and Nayak, 1979). *Calocybe indica* is rich in protein, mineral, fiber, carbohydrates, and is abundant with essential amino acids (Alam *et al.*, 2008). It is an excellent source of thiamine, riboflavin, nicotinic acid, pyridoxine, biotin and ascorbic acid. (Breene *et al.*,1990). People in Myanmar are still not very aware of nutritional and medicinal importance of mushrooms. The history of mushroom cultivation is very recent in the country. Thus, the present study was aimed to determine how the cultivation of mushrooms could improve rural livelihood, to cultivate them throughout the year.

## Material and Methods

### Collection of basal mycelium from milky mushrooms

Milky mushrooms were collected from Hlegu Township, Yangon Division. After the collection of the mushrooms, they were identified and classified according to literature cited by Krleger and Shaffer (1967) and Svrcek (1998).

### Selection of mushrooms for culture



stipe with small pileus  
(one week)



stipe with well differentiated  
pileus (two weeks)



Mature fruiting body  
(three weeks)

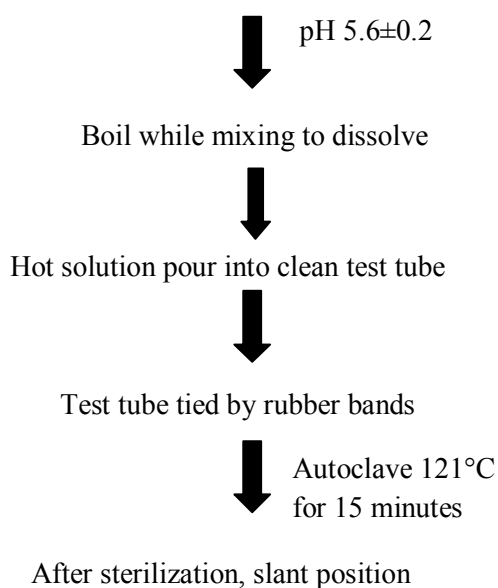
Figure. 1. Selected mushroom

### Culture media preparation by the method of Singh *et al.*, 2009

Material required: 30g of commercial PDA powder and 1000 ml distilled water, stirrer, beakers, aluminum pot, test tubes, cotton wool, paper, rubber bands and autoclave.

All the solutions were continuously stirred by a porcelain stirrer with regular speed until completely dissolved. The hot solutions were poured into cleaned test tubes to about one fourth of each test tube. Each test tube was plugged with a cotton wool and covered with a piece of paper (3cm in diameter). The plug has to be plugged tightly to keep the cotton stuffing in place and to prevent from wetting. After covering, the bottles were tied together by the rubber bands. Then, the bottles were placed in the up position in an autoclave at 15 psi and 121°C for 15 minutes to ensure complete sterilization. After the sterilizer had cooled down without opening the lid for about 30 minutes, the lid was opened so as to increase the surface area of the medium. The solution should come close to the neck but must not touch the cotton plug. After the PDA medium had slant position, it was left overnight to solidify.

39 g of commercial PDA powder +1L of distilled water



PDYA media = same above procedure + yeast 2g

Figure. 2. Flow chart of PDA medium

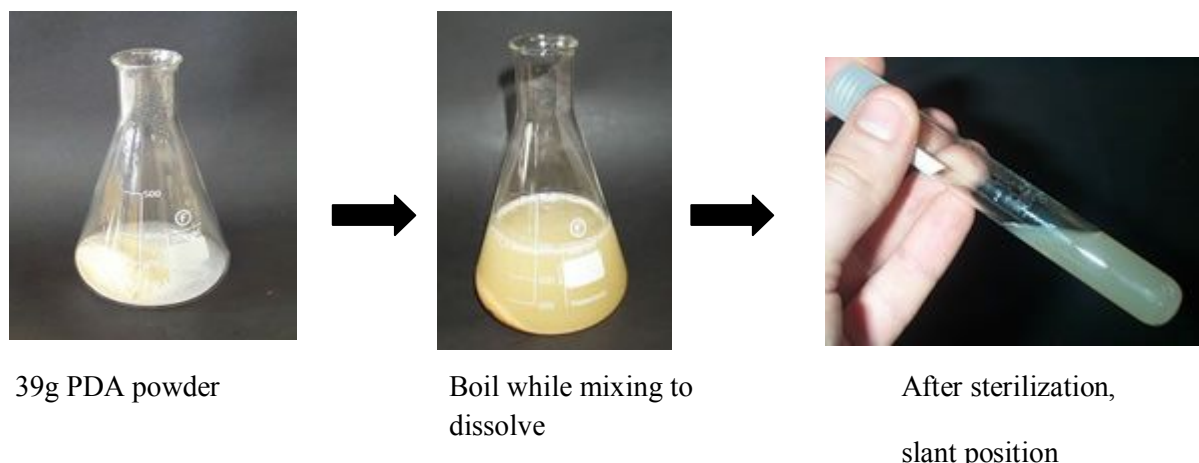


Figure. 3. Preparation of PDA medium

### Procedure of mushroom tissue culture

Fruit body of mushrooms (one week, two weeks, three weeks) were collected. The mushroom was surface sterilization with 70% alcohol for about one minute. The selected mushroom was cut by sterilized knife and a small piece of internal tissue was removed from the middle between the cap and the stalk of the mushroom. They were then inoculated onto the middle surface of the PDA and PDYA media. Immediately after inoculation, the mouth of the test tubes was flamed and plugged with cotton wool. After the tissue had been placed in the agar medium, the bottle was placed in an incubator at 27-31°C for mycelium growth. During this time, the mycelium growth rate was recorded every day.

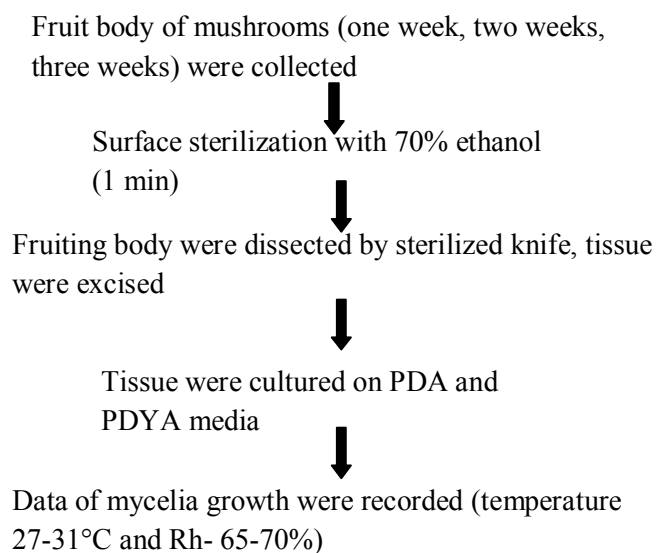


Figure. 4. Flow chart of mushroom tissue culture

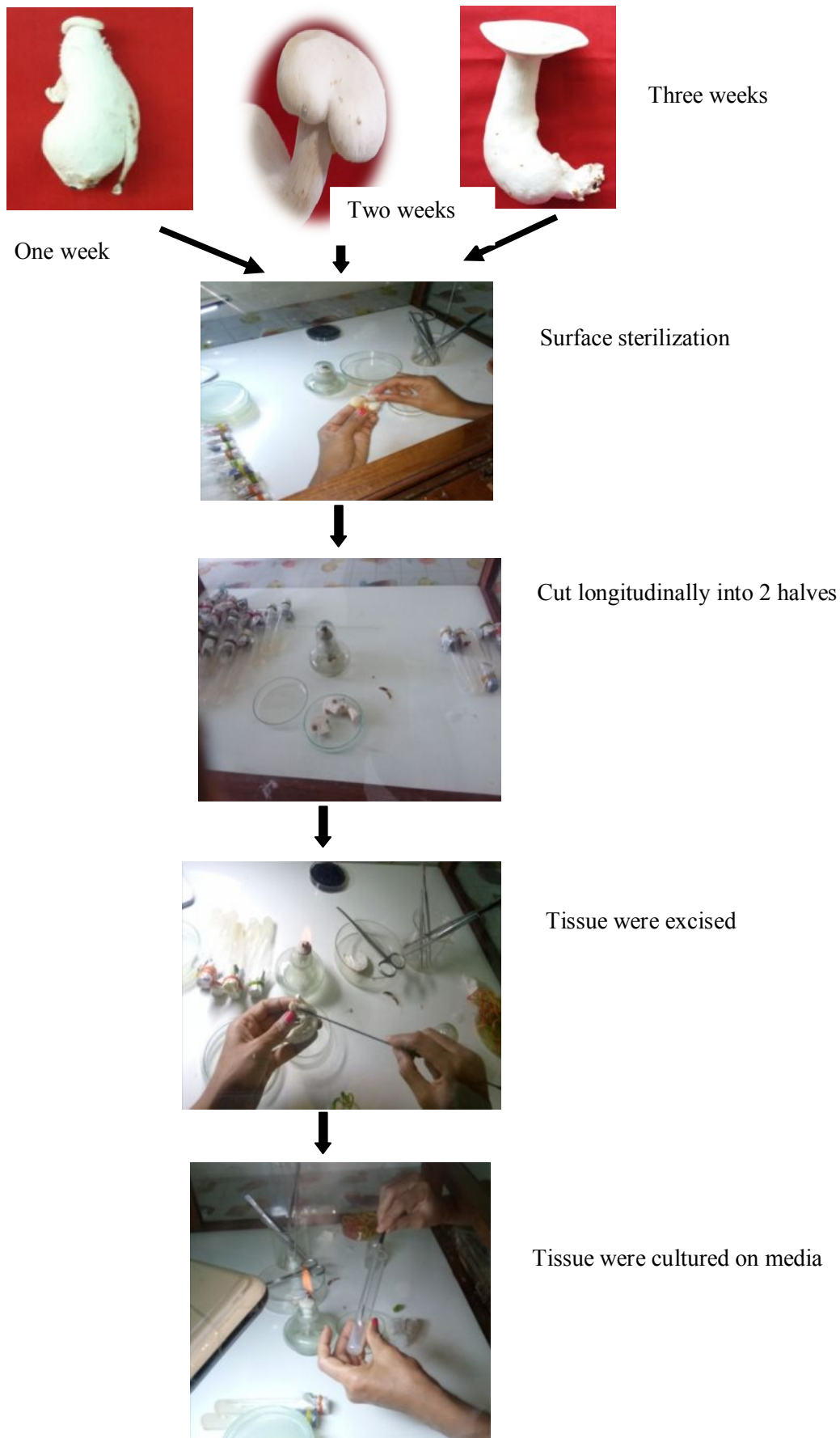


Figure. 5. Mushrooms culture on PDA or PDYA media

### Culture condition

All culture was inoculated at  $(27 \pm 3^\circ\text{C})$  under dark room and (65-70%) relative humidity. (Varshney , 2007)

### Measurement and recording of mycelium growth

The initial growth of mycelium was recorded by photographs under microscope. The growth of mycelium was measured in centimeter (cm) scale 2, 4, 6, 8,10,12,14 days and the data were recorded on mycelium growth. The average growth rate of mycelium in three replicates from one experiment was calculated.

### Preparation of mother spawn bottle by the method of Ram *et al.*, 2013

Sorghum grains or rice grains were washed and strained to remove all water. The grains were boiled in water till the skin started to crack. The boiled grains were drained and spread to cool down and to decrease moisture. They were then mixed with lime 0.5% + glucose 1%. The mixture was filled into about three fourth of clean bottles and each bottle was plugged with cotton wool, covered with paper piece and then tied together by a rubber band. Sterilization was carried out in autoclave at  $121^\circ\text{C}$  for 45 minutes. After the sterilizer had cooled down, the bottles with grains were transferred to the culture room.



Figure. 6. Preparation of mother spawn bottle



## Inoculation of mother spawn bottles by the method of Ram *et al.*, 2013

Material required:

- Bottles with sorghum or rice grains
- Flat bottles which are free from contamination and filled with pure mycelium
- The remainder of the laboratory equipments were the same as those of tissue culture

The inoculation of the bottles of spawn material with mycelium from the agar slant was made by using the procedure for inoculation of the slant with mushroom tissue. The needle was flamed till red hot and then cooled down. By the aseptic technique, the needle was used to cut out about one square centimeter of mycelium with agar medium in the flat bottle and it was taken under aseptic conditions as those of culture. All bottles were labeled to indicate the date and type of mushroom, incubated at  $23 \pm 2^\circ\text{C}$  in dark place. The growth rate of mycelium was recorded every day.

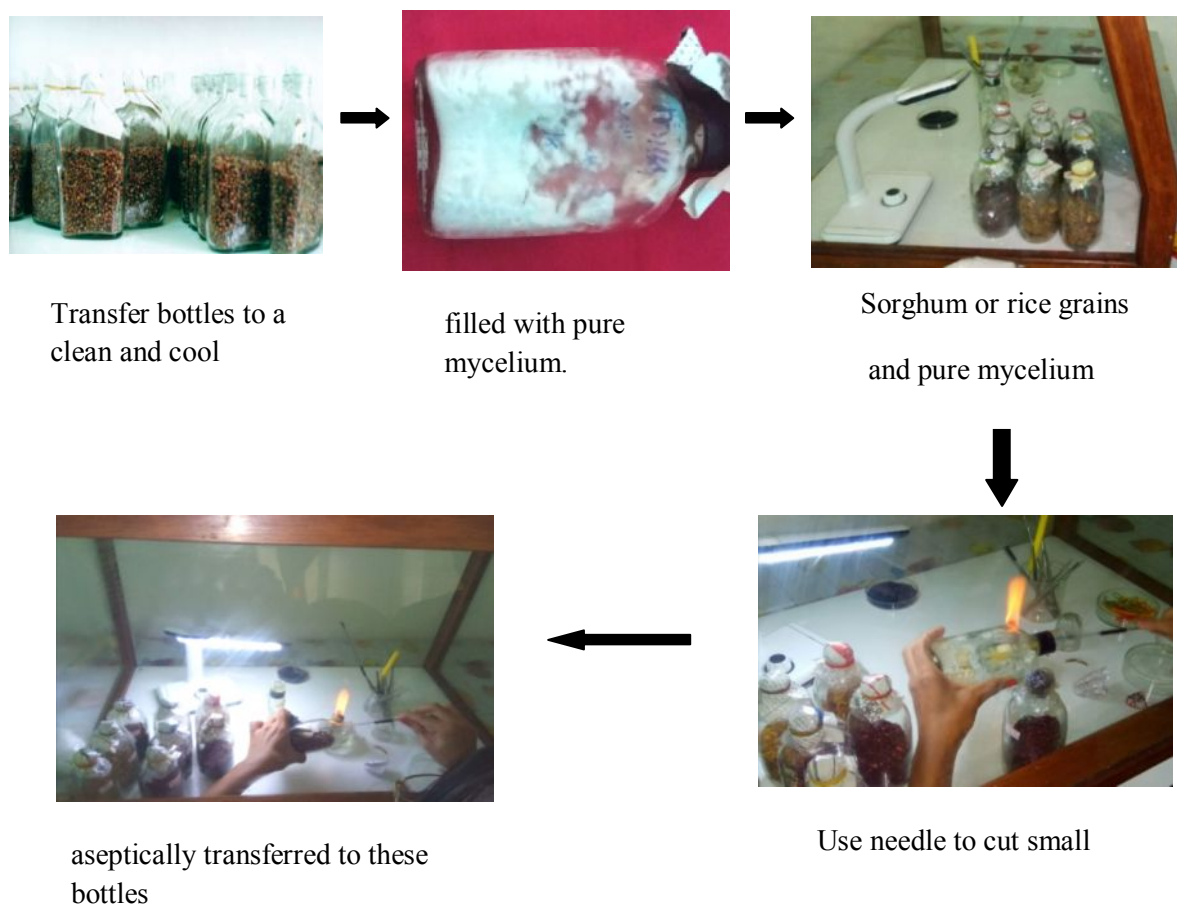


Figure. 7. Inoculation of mother spawn bottles

### **Preparation bag cultivation by the method of Baskaran *et al.*, 1978**

Make sure all ingredients are well mixed (Saw dust 94%+ rice bran 5% +lime 1%+ water). Then all the ingredients were thoroughly mixed well, moisture content between 65 - 75%. When a sawdust medium is grasped hard by hand, water might come out a little. The plastic bags were firstly folded to give a rectangular-shaped bottom; the thoroughly mixed ingredients were poured to about two third of specially prepared plastic bags and the bags were folded to enable the ingredients to be poured in easily and to keep the bags upright. The bags were lifted by the mouth and the bottom tightly tapped. The mouth of the plastic bag was grasped and pushed through the plastic collar and pulled out it to tauten the bags, then folded it over the plastic collar and bottle neck and tightly sealed with the rubber band. Then the stick or the pestle was bored into the center of the ingredients and made a hole almost to the bottom of the bag. Finally, the bag was plugged with cotton wool and covered with a piece of plastic and then finally tied together by the rubber band.

### **Sterilization of cultivation spawn bag**

A wooden rack was placed at the bottom of the oil drum of a height of around 20 cm. The drum was filled with water up to the height of the rack. The bags with the substrate were placed on the rack inside the oil drum. The lid was put on the drum and steamed for four to six hours in the drum with either charcoal. The steam was allowed to escape by a few small holes in the middle of the lid made by a 3" nail. When completed, the fire charcoal was taken out of the stove. It was then cooled down for approximated 30 minutes. The bags were taken off and cooled down more. The bags were then transferred to inoculation room.

### **Inoculation of cultivation bag**

#### **Material required:**

- Bag with rubber saw dust
- Bottle with sorghum grain which is free from contamination and filled with mycelium

The inoculation of cultivation spawn bag from the mother spawn bottle was made by using the procedure for inoculation of the slant with mushroom tissue. During the process of spawning the following measures need to be taken to control contamination of the substrate. The plug was removed and the bottles of sorghum or rice grains filled with mycelium was blended or stirred and vigorously shaken. The mouth of the grains bottles was flamed in order to prevent contamination. The other hand was use to remove the plug of the bag and about two tea spoonful



of mother spawn was poured into the bag and the plug was immediately replaced. The bag was close with the cotton as quickly as possible. After inoculation, all the bags were covered with paper to cover the top of the bag and tied with the rubber band.

The incubating room must be kept very clean and free of diseases to avoid contamination and also to avoid sunshine from entering the area. Cleanliness must be inspected before entering with new spawn bags. The bags can be placed horizontally or vertically. In this experiment, the bags were placed horizontally.



Figure. 8. Preparation and inoculation of spawn bag

## Results

### Morphological study

After collection of the mushroom, they were identified with the help of literature cited by Krieger and Shaffer (1967) and Svreek (1998).

### Morphological character of milky mushroom

Kingdom: Fungi

Division: Basidiomycota

Class: Agaricomycetes

Order: Agaricales

Family: Lyophyllaceae

Genus: *Calocybe*

Speices : *Calocybe indica* P&C

Common name: Milky mushroom

Habit: grows in grasslands, fields and generally on substrate rich in organic material.

Pileus: convex initially before flattening out with age. The cuticle (skin) can be easily peeled off the cap.

Gill: The crowded gills are white

Stipe: cylindrical stem

The flesh has a mild flavour.



Figure. 9. Habit of *Calocybe indica* P&C

### Effect of Potato Dextrose Agar (PDA) medium on mycelium growth

In Potato Dextrose Agar (PDA) medium, mycelium started on the entire inoculated tissue, T<sub>1</sub> (stipe with small pileus (one week)), T<sub>2</sub> (stipe with well differentiated pileus (two weeks)), T<sub>3</sub> (mature fruiting body (three weeks)).

In two days, T<sub>1</sub> is 0.1 cm, T<sub>2</sub> is 0.5 cm, T<sub>3</sub> is 0.3 cm. In four days, T<sub>1</sub> is 0.34 cm, T<sub>2</sub> is 0.73 cm, T<sub>3</sub> is 0.69 cm. In six days, T<sub>1</sub> is 0.5 cm, T<sub>2</sub> is 1 cm, T<sub>3</sub> is 0.8 cm. In eight days, T<sub>1</sub> is 1.12 cm, T<sub>2</sub> is 2.56 cm, T<sub>3</sub> is 2 cm. In ten days, T<sub>1</sub> is 1.54 cm, T<sub>2</sub> is 3 cm, T<sub>3</sub> is 2.32 cm. In twelve days, T<sub>1</sub> is 1.98 cm, T<sub>2</sub> is 3.5 cm, T<sub>3</sub> is 2.82 cm. In fourteen days, T<sub>1</sub> is 2.32 cm, T<sub>2</sub> is 4.17 cm, T<sub>3</sub> is 3.01 cm.

In these experiments, stipe with well differentiated pileus (two weeks) is highest growth with other treatments.

Table (1) Average mycelium growth of the different stage of mushrooms in Potato dextrose agar media (cm)

Days	Mycelium growth (cm)		
	T <sub>1</sub>	T <sub>2</sub>	T <sub>3</sub>
2	0.10	0.50	0.30
4	0.34	0.73	0.69
6	0.50	1.00	0.80
8	1.12	2.56	2.00
10	1.54	3.00	2.32
12	1.98	3.50	2.82
14	2.32	4.17	3.01

T<sub>1</sub> = stipe with small pileus (one week)

T<sub>2</sub> = stipe with well differentiated pileus (two weeks)

T<sub>3</sub> = mature fruiting body (three weeks)

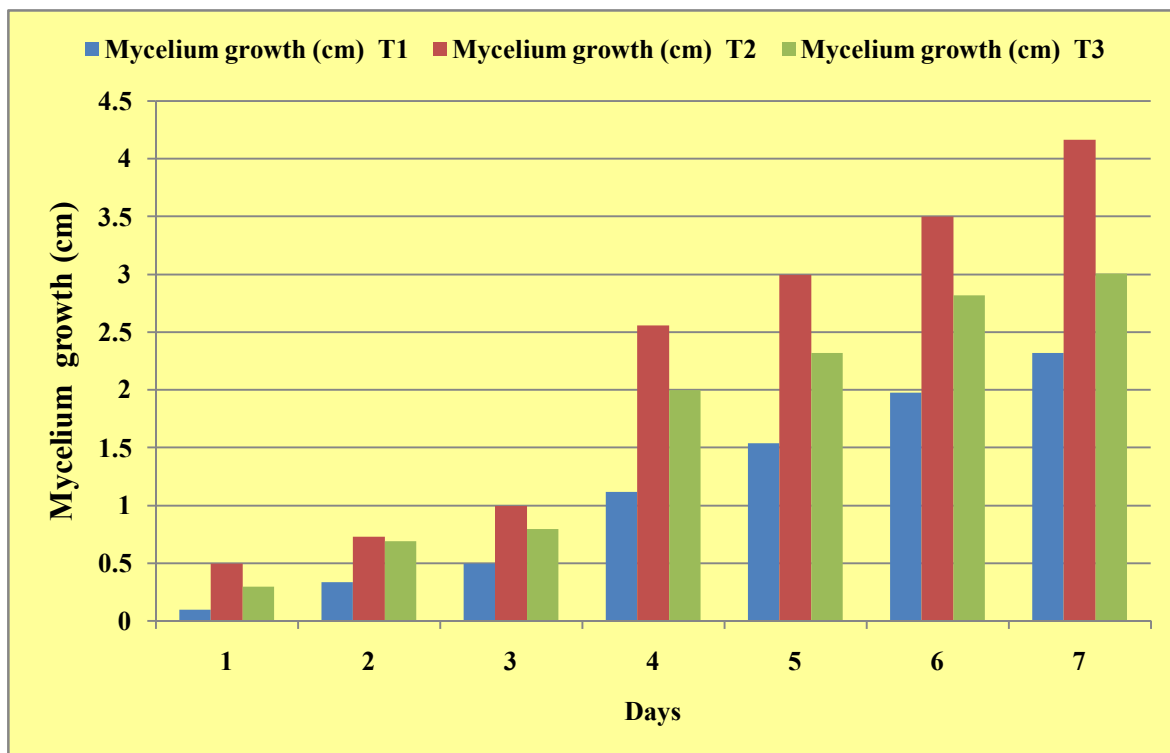
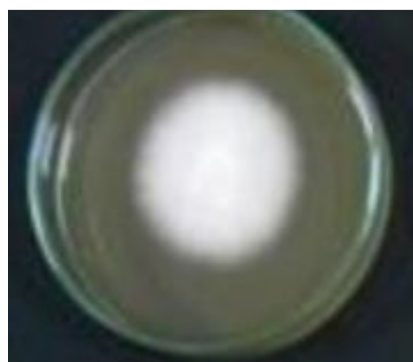


Figure 10 Comparison of mycelial growth milky mushroom cultured on PGA medium



T<sub>1</sub> = stipe with small pileus (one week)



T<sub>2</sub> = stipe with well differentiated pileus (two weeks)



T<sub>3</sub> = mature fruiting body (three weeks)

Fig . 11 . different stage of mushrooms in Potato dextrose agar media (Three weeks old culture )

### Effect of Potato Dextrose Yeast Agar (PDYA) medium on mycelium growth

In Potato Dextrose Yeast Agar (PDYA) medium, mycelium started on the entire inoculated tissue, T<sub>1</sub> (stipe with small pileus (one week)), T<sub>2</sub> (stipe with well differentiated pileus (two weeks)), T<sub>3</sub> (mature fruiting body (three weeks)).

In two days, T<sub>1</sub> is 0.53 cm, T<sub>2</sub> is 1.3 cm, T<sub>3</sub> is 1.09 cm. In four days, T<sub>1</sub> is 0.8 cm, T<sub>2</sub> is 1.86 cm, T<sub>3</sub> is 1.63 cm. In six days, T<sub>1</sub> is 0.5 cm, T<sub>2</sub> is 2.4cm, T<sub>3</sub> is 2.16 cm. In eight days, T<sub>1</sub> is 0.96 cm, T<sub>2</sub> is 2.5 cm, T<sub>3</sub> is 2.34 cm. In ten days, T<sub>1</sub> is 1.45 cm, T<sub>2</sub> is 3 cm, T<sub>3</sub> is 2.78 cm. In twelve days, T<sub>1</sub> is 1.98 cm, T<sub>2</sub> is 3.5 cm, T<sub>3</sub> is 3 cm. In fourteen days, T<sub>1</sub> is 2.32 cm, T<sub>2</sub> is 4.17 cm, T<sub>3</sub> is 3.32 cm.

In these experiments, stipe with well differentiated pileus (two weeks) is highest growth with other treatments.

Table (2) Average mycelium growth of the different stage of mushrooms in Potato dextrose agar media (cm)

Days	Mycelium growth (cm)		
	T <sub>1</sub>	T <sub>2</sub>	T <sub>3</sub>
2	0.53	1.30	1.09
4	0.80	1.86	1.63
6	0.50	2.40	2.16
8	0.96	2.50	2.34
10	1.45	3.00	2.78
12	1.98	3.50	3.00
14	2.32	4.17	3.52

T<sub>1</sub> = stipe with small pileus

T<sub>2</sub> = stipe with well differentiated pileus

T<sub>3</sub> = mature fruiting body

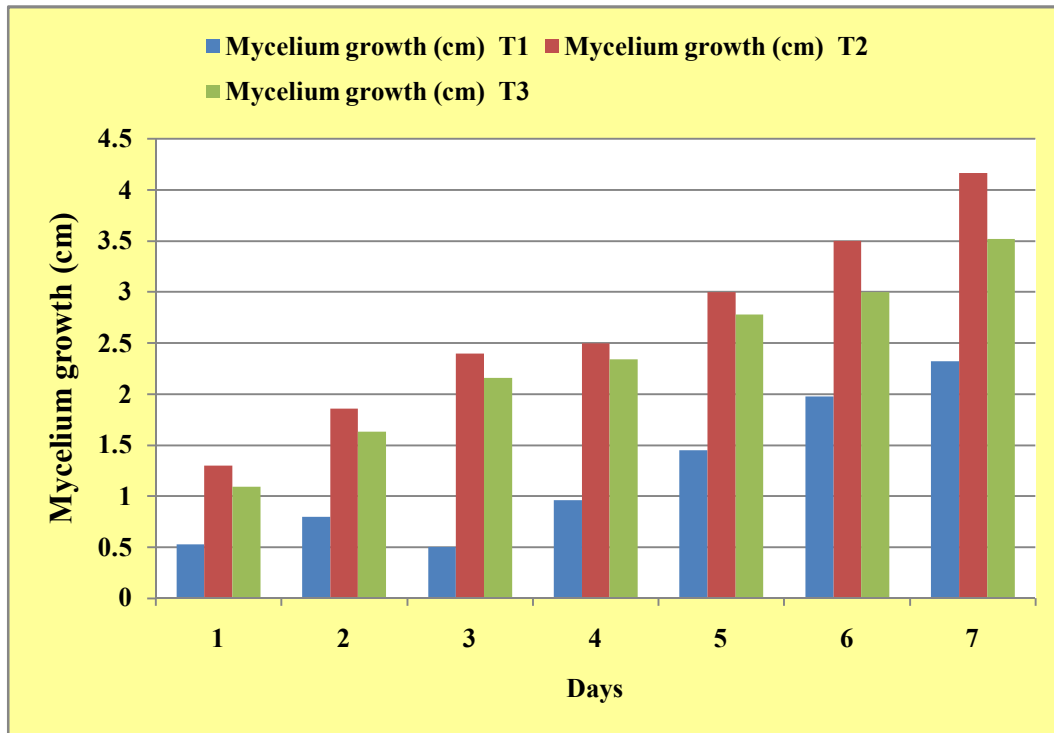
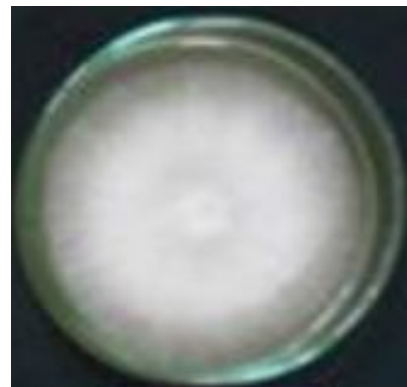


Figure 12 Comparison of mycelial growth milky mushroom cultured on PGYA



T<sub>1</sub> = stipe with small pileus (one week)



T<sub>2</sub> = stipe with well differentiated pileus (two weeks)



T<sub>3</sub> = mature fruiting body (three weeks)

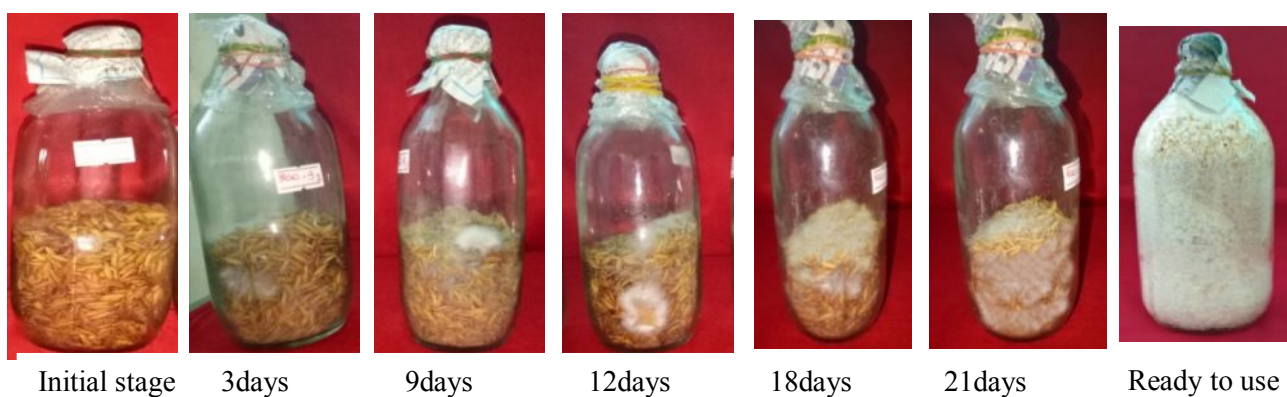
Fig . 13 . different stage of mushrooms in Potato dextrose yeast agar media (Three weeks old culture )



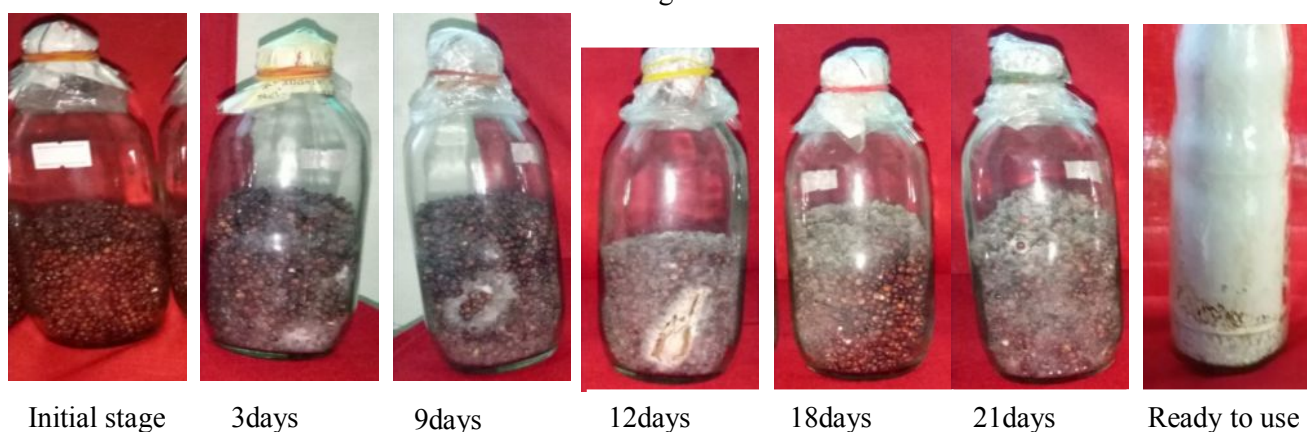
Table (3) Evaluation of different grain substrates for spawn development of *C. indica*

(one month)

	Complete spawn development by strains (days)*(cm)	Mycelial characteristics
Rice grain	28	White mycelial growth, all grains were covered. Grains were tightly held with each other by all strain.
Sorghum grain	23	White mycelial growth, all grains were covered. Grains were tightly held with each other by all strain.



Rice grains



Sorghum grain

Fig .14. Evaluation of different grain substrates for spawn development of *C. indica* (one month)





This stage placed in mature mycelium house



1½ months



When mycelium colonized the whole substrate thoroughly, the bags are carried to the fruiting room

20 days



10 days (fruiting body ready to harvest)

Fig .15. Growth and development of *C. indica*

## Discussion

In study, regarding cultural and the morphological characters of *Calocybe indica*, the size of the pileus of the fruiting body was convex at first later expanded and flattened, cuticle is easily peeled, mat polished, sometimes appressed scales present at or around the centre, margin regular in curved, smooth, non-striate. Stipe surface was dry and attached centrally, cylindrical with sub-bulbous base solid, white. Gill were distinctly formed crowded, emarginated, separable, white, unequal, thick, and attenuated towards margin. The cultural and morphological characters of the fruiting bodies of milky mushroom observed in the present study are similar to those described by Varshney (2007), Purkayastha and Chandra (1985). In the present investigation, a stipe with a well differentiated pileus (two weeks) in PDYA medium showed that the fastest mycelium growth of milky mushroom than the culture of one weeks (stipe with small pileus) and three weeks (mature fruiting body). The present finding is agreed with Pani (2016) who reported that the higher linear growth was achieved in response to the tissue culture obtained from the mushroom which consisted of a stipe with a well differentiated pileus in PDYA media. Krishnamoorthy *et al.*, (2015) reported that the majority of the time required for maximum mycelia growth in culture media like potato dextrose yeast agar media was 7 to 10 days than potato dextrose agar.

Senthilnambi *et al.*, 2011 conducted an experiment to find the suitability of different grains as spawn substrates and their effect on yield parameters of *Calocybe indica*. The results revealed the supremacy of sorghum grains as the most suitable substrate for early spawn run. Therefore spawn culture of the mushrooms is better suitable in sorghum grains. One bottle of pure culture on agar can produce ten bottles of subcultures, each of which can produce thirty to forty bottles of mother spawn and each of which can inoculate thirty five to fifty bottles of cultivation spawn bag.

According to this experiment, all media has been sterilized before they were used for inoculation and sterilization was done with steam and all experiments were conducted at room temperature of range from 25-34°C and 80-90% relative humidity in cultivation and planting of the spawn bag. The medicinal uses of milky mushrooms are low sugar level in blood and good circulation of blood. (Charles, 2010). Many products can be made with mushrooms, aside from being added to soups and sauces. Mushrooms can be converted into sweets, cookies, candies, various snacks, and can also be dried for later use.

Therefore, mushrooms cultivation may reduce poverty and improved the life style of many poor's people in our country and recycle agricultural wastes to cultivate mushrooms.

### **Conclusion**

- It is concluded that the edible mushrooms can be produced through tissue culture technique commercially.
- Mushrooms production depends on mycelia growth because the highest growth of mycelium may cause the highest yield of fruiting bodies.
- By means of applying the pure culture method, we can obtain the edible mushrooms of high quality on short period.
- Moreover, wastes such as cereal straws are largely burnt by the farmers, which cause air pollutions. However, these raw materials can actually be used for the cultivation of mushrooms.

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