

## Morphological and Molecular Characterization of Milky Mushroom *Calocybe indica* Mutants

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### ABSTRACT

Milky mushroom, *Calocybe indica* which is considered as the third most popular and commercially grown mushroom in Asia is the result of huge revolution in the mushroom technology in recent years with respect to types and strains of cultivated mushrooms. Mutation was carried out by applying gamma radiation with different doses (10,250,500,750 and1000gray) on *C. indica* mycelia grown on Potato Dextrose Agar (PDA). Post radiation observation on the mushroom morphology was detected. When measured 14 days after irradiation, strain 2 take less time to complete mycelia growth and mycelial growth of cultures treated with low dose (10 gray) was faster than that of unirradiated cultures. High doses (250, 500, 750, 1000 gray) inhibited mycelial growth significantly. As radiation dose increased over 250 gray, the growth parameter gradually decreased to reach a minimum at 1000 gray. Color and odor were not changed by irradiation. Mutants showed wide range of variability. In the present study, inter simple sequence repeat (ISSR) assays were used to identify DNA polymorphism among the mutant strains of *C. indica*, to identify DNA polymorphism among the mutants and non-treated (0.0gray) samples through ISSR assay, a total of 10 random primers were Screened, ISSR analysis showed high degree of variation yielding 58 bands including 40 polymorphic bands (39 shared and one unique band with molecular size 1050 pb in ST.1 by the dose 1000 gy). These findings indicated a medium level of genetic diversity among the studied *C. indica* strains and their mutants. The cluster analysis revealed three main clusters ,Cluster I consisted of only fourteen genotypes, cluster II consisted of three genotypes (CI-1.1000gy , CI-2.0gy and CI-2.10gy) while Genotype CI-1(500gy) revealed a completely different banding profile with all 10 selected primers and stood quite distinct from other genotypes in the final cluster .This study indicated that strains of *C. indica* and their mutants show abundant diversity in morphological and genetic diversity, and that DNA polymorphism detected by ISSR analysis offered a useful molecular marker for the identification of mutants in gamma radiation-treated mushroom .

**Key words:** Mushroom, ISSR, DNA Fingerprinting.

### Introduction

Since ancient time mushrooms have been used as food, especially in the eastern countries and recognized as natural and healthy foods (Iwalokun *et al.*, 2007). As time progress, there was increase in awareness about mushrooms as they are rich in proteins and fibers contain although contain low number of calories and fat (Khan *et al.*, 2011). Mushrooms are rich sources of nutraceuticals and their bioactive properties are already reported (Çaglarirmak, 2007 and Lindequist *et al.*, 2005). Earlier studies demonstrated antioxidant potential and antitumor, antibacterial, antiviral and hematological activity (Yang *et al.*, 2002 and Ribeiro *et al.*, 2006).

Milky mushroom (*Calocybe indica*) is relatively new to the world of mushroom industry. This mushroom was first reported from West Bengal, India (Purakayasha, 1974) (Purkayastha, 1984-85; Pandey and Tewari, 1993). Even though attempts were made to grow *C. Indica* (Purkayastha and Nayak, 1981; Pandey and Tewari, 1993) only limited success was achieved in increasing the bio-efficiency and productivity of this mushroom. Krishnamoorthy *et al.* (2003) reported a high yielding strain of *C.indica*, APK 2. It grows well at room temperature between 24°C-27°C and can be

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cultivated even on unfermented material. The mushroom can be harvested from 30-34 days after spawning. The advantages of this mushroom over other mushrooms are easy method of cultivation, less investment, very attractive fruiting body, pleasing milk white color, long shelf life, more nutritious and less time to grow. In general, Unique and rich nutritional status of Mushroom and growing demand for functional. (Bokaria *et al.*, 2014).

The Milky mushroom (*C. indica*) is a potentially new species to the world of mushroom growers. It is a robust, fleshy, milky white, umbrella like mushroom, which resembles button mushroom. The species is suitable for hot humid climate and can be cultivated indoor in high temperature and high humidity areas. It grows well at a temperature range of 25-35°C and relative humidity more than 80%. It is rich in protein, lipids, fibers, minerals, carbohydrate and contains an abundant amount of essential amino acids. It is an excellent source of thiamine, riboflavin, nicotinic acid, pyridoxine and ascorbic acid. Nutritive value of *C. indica* is comparable with other edible mushrooms (Zahid *et al.*, 2010).

Gamma rays come from spontaneous disintegration of radioactive nuclides (Cobalt 60 or Cesium 137) as their energy source (Mami *et al.*, 2013). They have short wave length, high energy photons, and have deep penetrating power. During irradiation, the radioactive nuclides are pulled out of storage (water pool) into a chamber with concrete walls that keep any gamma rays from escaping (Park and Vestal, 2002). Gamma irradiation technology promises to be a potential in this field in view of the fact that it has the ability to sterilize more compost bags per unit time, less laborious, more effective microbial reduction and hydrolytic agent (Gbedemah *et al.*, 1998).

Recently, DNA markers have become gradually more utilized for routine testing of the genetic diversity (Gilbert *et al.*, 1999). Various molecular marker techniques have been used to analyze the genetic diversity of edible mushrooms (Du *et al.*, 2012; Liu *et al.*, 2012; Pawliket *et al.*, 2012 and Anderson *et al.*, 2010). Inter Simple Sequence Repeat (ISSR) have been able to differentiate strains of a variety of homobasidiomycete mushrooms. ISSR described by Zietkiewicz *et al.* (1994) is a DNA marker determination tools based on PCR technique used in this study. The ISSR method has several advantages over other molecular marker methods; that is, DNA sequence information is not required before amplification, it is inexpensive, simple and highly stable, and it yields abundant genomic information (Bohn *et al.*, 1999; Camacho and Liston, 2001; Patzak, 2001; Behera *et al.*, 2008; Sabouet *et al.*, 2010). It can be used to identify species or varieties, to evaluate genetic diversity, in breeding programs, and for genomic finger printing (Bornet and Branchard, 2001; Joshi *et al.*, 2004; Reddy *et al.*, 2002; Thangavelu *et al.*, 2012; Zhang *et al.*, 2012).

The aim of this work was to investigate the response of three strains of milky mushroom *C. indica* to different doses of gamma irradiation (10, 250, 500, 750 & 1000 Gy) in terms of growth and yield. The ISSR markers have been investigated in the three strains following exposure to gamma radiation doses.

## Materials and Methods

### Collection of different varieties of mushroom

The first entry of these isolates in Egypt, as well as production and access to the fruits. The mushrooms (three strains *C. indica*) used in this study were purchased from a farm in Thailand from different regions; CI-1 from the Philippines is big flush.; CI-2 from India is less mushroom in flush but large size; CI-3 from Australia smaller size. The fungi were cultured on fresh Potato Dextrose Agar (PDA) medium for isolation, identification and characterization. The pathogen was purified and maintained by repeated sub-culturing after every month maintained at 25°C and kept in a refrigerator at 2 to 5°C for the following studies: The study was carried out at Genetics and Genetic Engineering Department collaboration with Plant Pathology department, Faculty of Agriculture, Benha University, Egypt.

### Sample preparation and irradiation

The mycelium of the three strains of mushroom *C. indica* of selected mycelia of (*C. indica*) were cultured onto potato dextrose agar (PDA) in petri dishes, incubated for 10 days and irradiated. The irradiation of (*Calocybe indica*) mycelia was carried out at were packed into petri dishes and

were exposed to five different doses of gamma radiation i.e. 10,250,500,750, 1000 Gy using dose rate 0.713 rad./sec.), per sec in air at room temperature (28±1°C). Doses were confirmed using the ethanol-chlorobenzene (ECB) dosimetry system at the Radiation a Cesium-137 gamma irradiator at Egyptian Atomic Energy Authority, National Center for Radiation Research & technology, Nasr City, Cairo, Egypt. All Petri dishes were incubated at 30°C for 7 days to examine mycelial colony morphology. Comparative yield performance of *Calocybe indica* parent and irradiated strains were assessed. The experiment was laid out as completely randomized design with three replications and five treatments.

Yield performance notices as: days for spawn run, days for case run, days for pins heads formation, No. of pinheads formed, No. of buttons harvested, days for first harvest, means No. of fruits/bed/500gm substrate, pileus diameter (cm), pileus thickness(cm),stipe length(cm),stipe breadth (cm), average weight (g), yield (g/500g) substrate, bio-efficiency B.E (%), biological efficiency of mushroom on fresh weight basis was calculated by formula given by Chang and Miles, (1989).

### **Mother spawn preparation**

Sorghum or wheat grains are used for spawn preparation. Half cooked grains, are mixed with calcium carbonate @ 20g per kg of grains (dry weight), thoroughly mixed and filled in polypropylene bags (15x30 cm size) provided with PVC rings as neck. The bags are tightly plugged with non-absorbent cotton and sterilized at 1.42-kg/cm<sup>2</sup> pressure and 126 °C temperature for 1.5-2.0 hours in an autoclave. When the bags are cool, they are aseptically inoculated with fresh cultures of oyster mushroom fungus. The work should be done in a culture room or in a laminar flow chamber. After inoculation, the spawn bags are stored in a clean room for 15-20 days before use. These bags with white mycelial growth serve as mother culture. Each mother spawn bag can be used for inoculating 30 bed spawn bags that can be prepared following the above procedure.

### **Mushroom bed preparation**

Polythene bags of 60x30 cm or 75x45 cm size are used for bed preparation. Chaffed paddy straw bits of 3-5 cm length are soaked in cold water for 4-5 hours. After draining the excess water, the straw bits are boiled for 45-60 minutes in a separate drum. Though hot water treatment is the safe and best method of sterilization, steam treatment or chemical treatment with a solution containing carbendazim and formalin can also be followed. After treatment, the substrate is shade dried to remove excess moisture before bed preparation. At the time of bed preparation, the substrate should contain around 60%moisture (can be tested by squeeze method). Cylindrical beds are prepared following layer method of spawning. A layer of straw is laid and sprinkle one tablespoon full of spawn over the filled straw around the peripheral region. A second layer of processed straw is filled and spawned as above. Repeat the process until the soaked straw is finished. Every time before spawning, press the straw with hand for making it compact. Finally, the bag is close tightly with twine and the beds are incubated for spawn running under semi-dark condition in a clean room. Spawn run will be completed in 12-15 days at 30-35°C.

### **Casting**

Milky mushroom production involves an additional process called casing. After the completion of spawn run, the cylindrical beds are cut horizontally into two equal halves. Apply casing soil on to both halves to a height of 1-2 cm. The casing soil is prepared by steaming garden soil (clay loam, pH around 8.0) for one hour.

### **Cropping**

After casing the beds are to be incubated over racks in a partially sunken chamber lined with blue colored high density polythene sheet as roofing material. Optimum relative humidity of 80-95%, room temperature of 24-28°C and light intensity of about 1600-3200 lux. should be maintained in the cropping room. Proper ventilation for gaseous exchange is also essential in this chamber. The beds are

regularly sprayed with water to maintain 50-60% moisture level on the casing surface. Pinheads appear in 8-10 days after casing and the first harvest can be made in 6-8 days after pinhead formation. After obtaining the first harvest the casing medium is gently ruffled, slightly compacted back and sprayed regularly with water. Second and third harvest may be obtained within 45-50 days of bed preparation. Then the beds are removed and fresh beds may be kept.

### **Mushroom harvesting and weighing**

The milky mushroom (*C. indica*) is a potentially new species to the world mushroom growers. It is a robust, fleshy, milky white, umbrella like mushroom, which resembles button mushroom. The species is suitable for hot humid climate and can be cultivated indoor in high temperature and high humidity areas. It grows well at a temperature range of 25-35 °C and relative humidity more than 80%. Milky mushrooms can be cultivated throughout the year in the entire plains of India. The cultivation technology is very simple, involves less cost and no special compost is needed for the cultivation. The cultivation process resembles that of oyster mushroom but for the additional process of casing. The mushroom can be harvested from 24-28 days after spawning and the total crop cycle is only 45-50 days. Most importantly, the milky mushroom has an extended shelf life of 3-5 days compared to other cultivated species, making it more amenable to handling, transportation and storage. So, there is a growing interest among the farmers towards milky mushroom. The production technology of milky mushroom is outlined here: Substrates Milky mushroom can be cultivated on a wide range of substrates like, paddy straw, maize stalks, sorghum stalks, pearl millet stalks, palmarosa grass, vetiver grass, sugarcane bagasse, soybean hay, groundnut haulms etc. However, for commercial production paddy straw is the best substrate.

### **Cropping and harvest**

After 20-22 days, when bags are fully impregnated with white mycelium, transfer the bags into cropping room and remove polythene/polypropylene covers. The open blocks should be kept in racks about 20cm apart. Rack should be 60 cm wide with gap of 50-60 cm between two shelves. Mushrooms grow in a temperature range of 20-33°C. Relative humidity is maintained by spraying water twice a day on the walls and floor of the room. Spraying of blocks should be avoided for the first 2-3 days. A light mist spray of water is given on blocks as soon as the small pin heads appear. Once pinheads are 2-3 cm big a little heavier watering is to be done on blocks and further watering of blocks is to be stopped to allow them to grow. Mushrooms should be plucked before they shed spores to maintain quality. After 1<sup>st</sup> flush of harvest, 0.5 to 1 cm outer layer of the block should be scrapped. This helps to initiate 2<sup>nd</sup> flush which appears after about 10 days. After harvest, the lower portion of the stalk must be cleaned with dry cloth. They should be packed in perforated (5-6 small holes) polythene bags to keep them fresh. It loses freshness after about 6hours, which can be enhanced by keeping them in refrigerator. Oyster mushroom can be sun dried for 2 days and dried product marketed in polythene bags. Dried mushrooms should be soaked in water for 10 minutes before use (Josephine and Sahana, 2014).

### **Production and isolation of suspected mutant**

The mycelium of the three strains of *C. indica* of mushroom Petri dishes (9cm) were packed into polythene containers and were exposed to five different doses of gamma radiation i.e. 10, 250, 500, 750, 1000 Gy using dose rate (0.713 rad per sec). The suspected mutant colonies were sub-cultured on PDA 9cm petri dishes for 3 times to study genetic instability of mushrooms as affected by continuous sub-culturing for mycelium growing & were sub-cultured on PDA slants as stock cultures. The irradiated mycelia were diluted serially with the mixture of 0.01% Tween 20 and 0.85% NaCl. Each 0.1 ml of optimum dilution was spread on PDA plate and duplicate plates were done. All plates were incubated at 30°C for 7 days to examine mycelial colony morphology. The suspected mutant colonies were sub-cultured on PDA slants as stock cultures. Comparative yield performance of *C. indica* parent and irradiated strains: The experiment was laid out as completely randomized design with three replications and five treatments. All parameters were taken as mentioned before.

## Genetic diversity through ISSR analysis

### DNA extraction

Three strains of the mushroom *C. indica* (CI-1, CI-2, CI-3) Fruits were collected in 1.5 ml Eppendorf tube, quickly frozen in liquid nitrogen and ground with konte pestles into fine powder. DNA was extracted according to Doyle and Doyle, (1990) mini preparation protocol.

### ISSR- PCR

ISSR-PCR was carried out according to Williams *et al.* (1990). The primers used were 11 to 18 mer oligonucleotide; ten primers were selected as potentially useful. The codes and sequences of the used primers are shown in Table (1). PCR reactions were optimized and mixtures (25 µl total volume) were composed of dNTPs (200 µM), MgCl<sub>2</sub> (1.5 mM), 1x buffer, primer (0.2 µM), DNA (50 ng), Taq DNA polymerase (2 units). Amplification was carried out in a thermo cycler programmed for 94°C for 3 min (one cycle); followed by 94°C for 30 sec, 40°C for 45 sec and 72°C for 1 min (35 cycle), 72°C for 10 min (one cycle) then 4°C (infinite).

Amplification products (25 µl) were mixed with 3 µl loading buffer and separated on 1.3% agarose gel and stained with 0.5 µg/ml ethidium bromide, and visualized under ultraviolet light and photographed. DNA fragment sizes were determined by comparisons with the 1 kb DNA ladder marker (Table, 1).

**Table 1:** Name and sequences of the used primers with ISSR molecular markers.

ISSR Primer	Nucleotide sequence 5' to 3'
814 <sub>A</sub>	(CT) <sup>8</sup> TG
844 <sub>A</sub>	(CT) <sup>8</sup> AC
844 <sub>B</sub>	(CT) <sup>8</sup> GC
17898 <sub>A</sub>	(CA) <sup>6</sup> AC
17899 <sub>A</sub>	(CA) <sup>6</sup> AG
HB <sub>9</sub>	(GT) <sup>6</sup> GG
HB <sub>11</sub>	(GT) <sup>6</sup> CC
HB <sub>12</sub>	(CAC) <sup>3</sup> GC
HB <sub>13</sub>	(GAG) <sup>3</sup> GC

### Data analysis

The obtained data of ISSR analysis was entered in a computer file as binary matrices where "0" stands for the absence of a band and in each individual sample. Similarity coefficients were calculated according to dice matrix (Nei and Li, 1979; Rohlf, 1993).

## Results and Discussion

### Effect of different gamma irradiation levels on growth rate of *C. indica* mycelia strains *in vitro*

The growth rate of Gamma irradiated *C. indica* mycelium on PDA is shown in (Fig. 1). The effect of radiation on growth rate of the irradiated mycelium was clearly observed after 14th days. The growth rate of irradiated mycelia strain 2 take less time to complete mycelia growth following by strain 3 as mean. Mycelial growth of cultures treated with low dose (10 gray) was faster than that of unirradiated cultures while high doses (250, 500, 750, 1000 gray) inhibited mycelial growth significantly. Mutant CI-2 (10 gray) take short time to complete mycelium growth (9.00 days). As radiation dose increased over 250 gray the growth parameter gradually decreased to reach a minimum at 1000 gy. Color and odor were not changed by irradiation. This method is in line with the statement of Esser (1971) who reported that the effective method for radiating mushroom is by applying the mutagenic agents to mycelia grown on agar media. These results established by (Djajanegara, and

Harsoyo, 2008) by observing the rate of mycelia growth reflected by the diameter of mycelia after radiation, their results notified that mycelia growth of mutant PO-5 and control of white oyster mushroom differs significantly starting at 4 days' post radiation. The significant difference was observed until the mycelia covered the entire plate, which was on 12 days' post-radiation. only PO-5 showed faster mycelia growth compared to control (po-K).

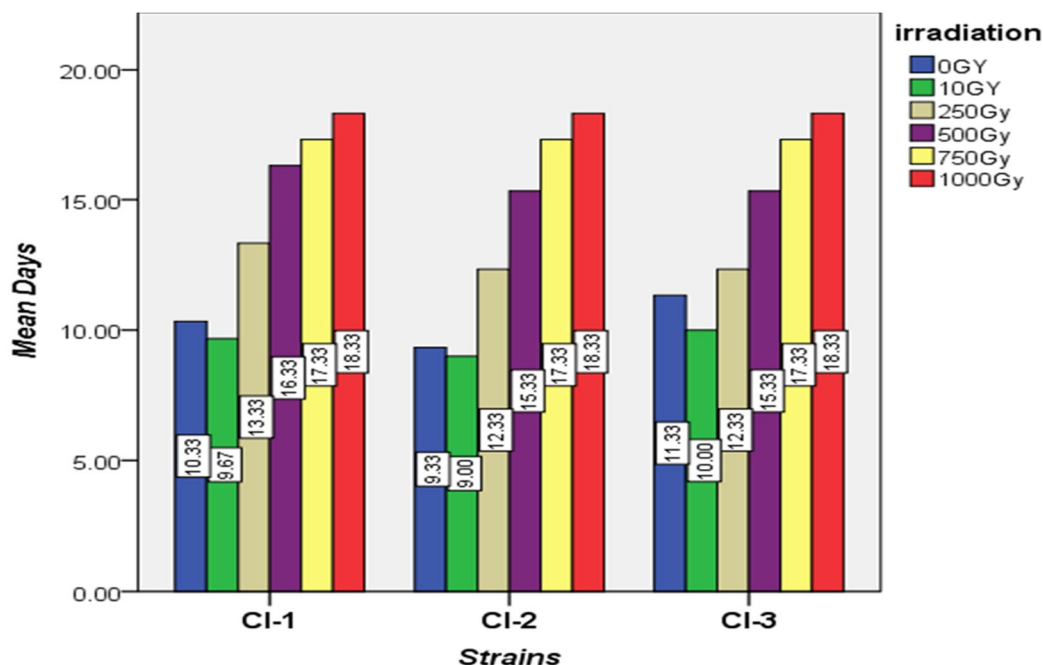


Fig. 1: Effect of Gamma different irradiation levels on growth rate of *Calocybe indica* mycelia strains *in vitro*.

#### Effect of different gamma irradiation levels of *Calocybe indica* mycelia strains *in vivo*

The results offered important variations between *C. indica* strains under study and between six doses of gamma rays, as well as the interaction between them (Table 2) for yield and morphological performance, i.e.; days for spawn run, days for case run, days for pinheads' formation, NO. of pinheads formed, NO. of buttons harvested, days for first harvest, pileus diameter (cm), pileus thickness (cm), stipe length (cm), stipe breadth (cm), yield (g/500g substrate) & bio-efficiency (B.E.%). Average growth performance of *Calocybe indica* mutants and their control were shown in (Table 2). The strain CI-1 gave the highest yield, B.E %, the smallest cultivation period & the largest no. of fruits so it was the best strain (Table 2). Irradiation dose 750 gy gave the highest yield, B.E. % & the largest no. of fruits bodies (Table 2). Mutants CI-1(250gy), CI-1(500 gy), CI-1 (750 gy) & CI-1(1000gy) gave high yield & B.E% compared to the control while mutant CI-1(10 gy) gave yield and B.E.% less than the control. mutants CI-2(250gy), CI-2(250 gy), CI-2(500gy) & CI-2(750 gy) gave high yield, B.E %, short cultivation period compared to the control while mutant CI2(1000 gy) gave yield, B.E % less than the control. mutants CI-3(10 gy), CI-3(250 gy), CI-3(500 gy), CI-3(750 gy) & mutant CI-3(1000 gy) gave high yield, B. E%, compared to the control. Mutant CI-1(250 gy) gave the highest yield (746.67 g), B.E %(149.33%) & the highest no. of buttons (12.67) following by mutant CI-1(750 gy) with high yield (741.70) and B.E % (148.33%) (Table2). Accordingly, these results confirmed that mutants CI-1 (250 gy) & CI-1 (750) is very important in next breeding and production programs for milky mushroom (*Calocybe indica*). These results established by many searches: (Tharcker, 1999) decided that using gamma irradiation may cause some mutations to the genes of cells through the DNA repair mechanisms within cells. (Reitsma-Wijkeret *al.*, 2000) proposed that the mutational spectrum depended on irradiation conditions and DNA repair mechanisms of the base excision repair are the causes of the mutational spectra in the target genes. Similar feedback was also reported (Djajanegara and Harsoyo, 2008) that mutant strain of oyster mushroom (irradiated at 0.75 kGy) showed significantly higher productivity compared to control.

Table 2: Effect of different irradiation levels of *Calocybe indica* mycelia strains *in vivo*.

Strain	Irradiation dose (gy)	DSR**	DCR	DPF	NPF	NBH	DFH	PD	PT	SL	SB	Y	B. E
CI-1	0 (control)	14.00	9.33	23.33	16.33	5.67	32.00	11.83	3.50	10.17	4.23	360.00	72.00
	10	14.33	12.33	26.67	21.33	10.67	42.67	7.83	2.27	5.90	2.47	343.30	68.67
	250	15.33	10.00	25.33	56.00	12.67	37.67	14.50	4.00	7.63	3.97	746.67	149.33
	500	15.67	10.67	26.33	38.00	9.00	38.00	16.00	3.50	6.00	4.40	660.00	132.00
	750	16.00	11.33	27.33	42.67	10.00	39.67	15.50	3.77	7.00	3.23	741.70	148.33
	1000	16.33	10.00	26.33	31.33	8.00	38.67	13.33	3.60	7.27	3.27	570.00	114.00
CI-2	0 (control)	14.00	12.67	26.67	25.33	6.00	42.67	9.00	2.63	7.67	2.93	318.33	63.67
	10	13.67	13.67	27.33	30.33	7.33	42.33	10.00	2.40	7.43	2.27	341.70	68.33
	250	15.67	11.33	27.00	18.67	7.00	35.00	13.83	3.50	7.50	3.03	441.70	88.33
	500	13.33	11.67	25.00	15.00	8.00	32.33	14.83	3.57	7.60	3.50	491.70	98.33
	750	16.00	11.67	27.67	23.00	9.67	39.33	9.83	2.90	6.50	2.60	491.70	98.33
	1000	16.33	12.33	28.67	27.00	6.00	41.33	8.00	3.17	5.70	2.43	315.00	63.00
CI-3	0 (control)	14.33	18.33	32.67	16.00	4.00	50.67	7.50	2.50	4.00	2.17	300.00	60.00
	10	14.67	17.67	32.33	13.00	5.00	52.33	7.27	2.57	3.50	2.23	325.00	65.00
	250	15.33	16.67	32.00	22.00	3.33	53.33	7.67	2.10	8.00	3.53	303.30	60.67
	500	13.33	18.33	31.67	8.00	4.67	50.33	9.00	2.37	7.00	3.50	350.00	70.00
	750	15.67	14.67	30.33	6.67	4.33	51.33	12.00	1.47	5.10	4.90	346.67	69.33
	1000	16.00	16.67	32.67	7.33	3.67	50.33	10.00	3.40	5.83	2.70	331.67	66.33
LSD 0.05		NS	1.37	1.63	10.15	2.6	1.58	2.52	0.64	1.19	0.65	44.6	8.92

\*\* Where DSR is Days for spawn run, DCR is Days for case run, DPF is Days for pinheads' formation, NPF is NO. of pinheads formed, NBH is NO. of buttons harvested, DFH is Days for first harvest, PD is Pileus diameter (cm), PT is Pileus thickness (cm), SL is Stipe length (cm), SB is Stipe breadth (cm), Y is Yield (g/500g substrate) & B.E. % is Bio-efficiency.

## Molecular screening using inter sequence repeat polymerase chain reaction analysis (ISSR-PCR)

### ISSR-PCR analysis

After screening, 10 ISSR primers were selected that produced clear bands with good polymorphisms and reproducibility. The ISSR bands were scored as 1 for presence and 0 for absence of markers for estimating genetic variation as a result of gamma-radiation. The ten ISSR primers amplified 58 bands, including 40 polymorphic bands. The results revealed one unique band with molecular size 1050 pb in CI-1 by the dose (1000 gy). The ISSR profiles for the CI genotypes are illustrated in Figures from 1 to 10. The total number of bands amplified by single ISSR primer ranged from 4 to 8 with an average of 5.8. The proportion of polymorphic bands ranged from 40% (primer 44B and HB- 13) to 100% (primer 49A) with an average of 64%. The size of the detected fragments ranged from 230 pb and 1250 pb only one of 10 primers revealed ISSR loci with 100% polymorphism (49A), other primers detect polymorphism loci from 40%(44B) to 87.5 (HB-09). The PIC and MI values are shown in Table 3. The PIC value provides an estimate of the discriminatory power of the ISSR marker to detect polymorphism. The PIC values for markers in the present study ranged from 0.10 for primer HB-15 to 0.356 for primer 49A, with a mean value of 0.23. These findings indicated a medium level of genetic diversity among the studied *Calocybe indica* strains and their mutants. The MI for ISSRs ranged from (0.22 to 2.135) and the mean MI per ISSR was 1.055. Primer 14A revealed 4 bands ranging in size between 250 to 1250 pb. Two bands were polymorphic and two band was monomorphic (Fig. 2 and Table 3). The lowest number of bands (2) was scored in CI-2 by the dose (250, 1000 gy) and in CI-3 by the dose (0, 250, 500, 750 gy) while the highest number of bands four was scored in CI-1 by the dose 1000gy of gamma radiation and in CI-3 by the dose of 10gy. The PIC for this primer was 0.229 and MI was 0.458 (Table 3). Primer 44B revealed five bands ranging in size between 320 to 820 pb; two bands were polymorphic and three band was monomorphic (Fig. 2 and Table 3). The lowest number of bands Three was scored in the control of CI-1 strain, while the highest number of bands five was scored in CI-1 control, by the dose (500 and 750 gy) of gamma radiation and in CI-2 strain by the dose of (250, 500 and 1000 gy) and in CI-3 by the dose of (10 and 500 gy). The PIC for this primer was 0.168 and MI was 0.336 (Table 3). Primer 49A revealed 6 bands ranging in size between 300 to 930 pb; all of them were polymorphic (Fig. 2 and Table 3). The only one band was scored in CI-2 strain by the dose 1000gy while the highest number of bands (6) was found in st.1 by the dose 1000 gy, CI-2 by the dose (250 and 500 gy) and CI-3 by the dose 1000gy of gamma radiation and polymorphic information content (PIC) for this primer was 0.356 and MI was 2.135 (Table 3). Primer 98B revealed 8 bands ranging in size between 280 to 1080 pb; six bands were polymorphic and two

bands was monomorphic (Fig. 2 and Table 3).

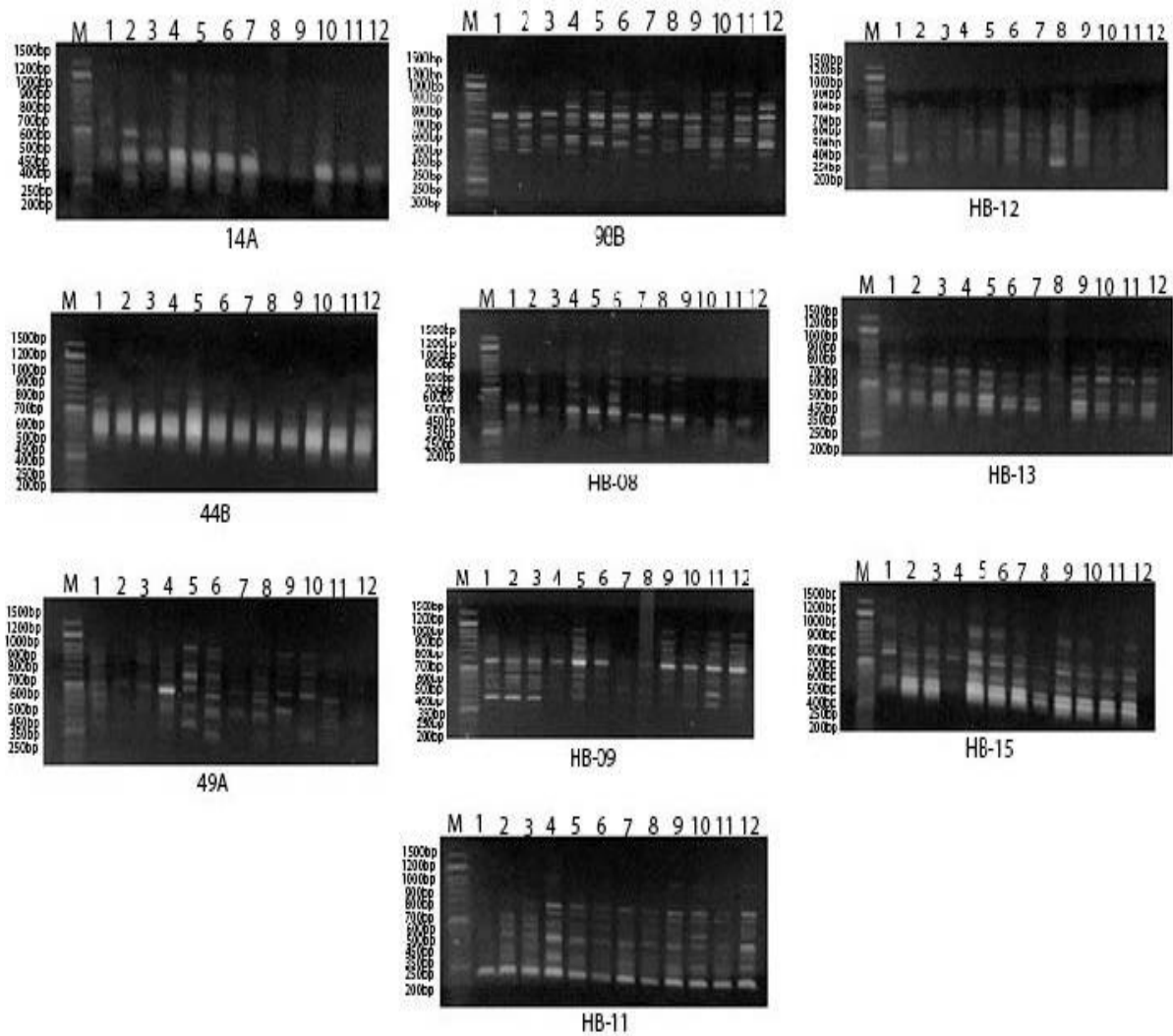


Fig. 2: Genetic diversity between all strains milky mushroom through ISSR analysis.

Table 3: Molecular profiles of the ISSR primers, used for DNA fingerprinting of the mutant lines and parents.

Primers	Sequence (5'-3') with repeat motif	No. of scored bands	Size range (approx. in bp)	Polymorphic Bands (B)			Mono- morphic bands	% Poly- morphism(B/Na)	PIC value	Marker Index(MI)
				Shared (S)	Unique (U)	Total Polymorp hic Bands (T)				
814 <sub>A</sub>	5'-CTCTCTCTCTCTCTTG- 3'	4	250-1250	2	0.0	2	2	50.00	0.229	0.458
844 <sub>B</sub>	5'CTCTCTCTCTCTCT GC 3'	5	320-820	2	0.0	2	3	40.00	0.168	0.336
17898 <sub>A</sub>	5' CACACACACACAAC 3'	6	300-930	6	0.0	6	0	100.00	0.356	2.135
17899 <sub>A</sub>	5' CACACA CACACAAG 3'	8	280-1080	6	0.0	6	2	75.00	0.259	1.550
HB-08	5' GAGAGAGAGAGAGG 3	5	380-1050	2	1.0	3	2	60.00	0.274	1.100
HB-09	5'-GTGTGTGTGTGTGC-3'	8	270-960	7	0.0	7	1	87.50	0.335	2.350
HB-11	5'-GTGTGTGTGTGTCC- 3'	7	230-1050	5	0.0	5	2	71.43	0.264	1.590
HB- 12	5'-CACCACCACGC-3'	4	340-800	2	0.0	2	2	50.00	0.205	0.410
HB- 13	5'-GAGGAGGAGGC-3'	5	420-840	2	0.0	2	3	40.00	0.110	0.220
HB- 15	5'GTGGTG GTG GC3'	6	350-960	4	0.0	4	2	66.66	0.100	0.400
Total		58.0		38	1	39.0	19	-	-	-
Minimum		4.0		2.0	1	2.0	0	40.0	0.100	0.220
Maximum		8.0		6.0	1	7.0	3	100.0	0.356	2.135
Mean		5.8		3.8	1	3.9	1.9	64.0	0.230	1.055



The lowest number of bands (4) was scored in the control of CI-1 and CI-2 mutant Isolates by the dose (10 and 250 gy) while the highest number of bands (8) was scored in CI-2 mutant isolate by the dose 500gy of gamma radiation and in CI-3 mutant isolate by the dose of 250gy. The PIC for this primer was 0.259 and MI was 1.55 (Table 3).Primer HB-08 revealed five bands ranging in size between 380 to 1050 pb; four bands were polymorphic (three is shared and one is unique with molecular size 1050 pb in CI-1 mutant isolate by the dose 1000gy) and only one band was monomorphic (Fig. 3 and Table 3). The only one band was scored in CI-1 control and mutant isolate by the dose (0 and 250 gy), CI-2 strain by the dose (0 and 500gy) and CI-3 control and mutant isolate by the dose (0,10,250 and 500 gy) while The highest number of three bands was scored in CI-1 mutant isolates by the dose (10,500,750,1000gy) of gamma radiation, CI-2 mutant isolates by the dose of (10, 250gy) and CI-3 mutant isolates by the dose of (750, 1000gy). The PIC for this primer was 0.274 and MI was 1.1 (Table 3).Primer HB\_09 revealed nine bands ranging in size between270 to 960 pb; seven bands were polymorphic and one band was monomorphic (Fig. 2 and Table 3). The only one band was scored in CI-1 mutant isolate by the dose 500gy while the highest number of bands nine was scored in CI-1, CI-2 mutant isolates by the dose 750gy of gamma radiation and CI-3 Mutant isolates by the dose of (10,250,500,750gy); The PIC for this primer was 0.335 and MI was 2.349 (Table 3).Primer HB-11 revealed seven bands ranging in size between 230 to 1050 pb; five bands were polymorphic and two band was monomorphic (Fig. 2 and Table 3). The lowest number of bands two was scored in the control of CI-1 while the highest number of bands six was scored in CI-1mutant isolate by the dose 500gy of gamma radiation, CI-2 by the dose of 250,1000gy and CI-3 mutant isolate by the dose of (10,1000gy); the PIC for this primer was 0.264 and MI was 1.6(Table 3).Primer HB-12 revealed four bands ranging in size between 340 to 800 pb; two bands were polymorphic and two bands was monomorphic (Fig. 2 and Table 3). The lowest number of bands two was scored in CI-1 mutant Isolates by the dose (10,250,500and750gy), CI-1 mutant Isolates by the dose (500,750and1000gy) and CI-1 mutant isolates by the dose of (0,250and750gy), while the four bands was scored in CI-1 mutant isolate by the dose 1000gy of gamma radiationand CI-2 mutant isolates by the dose of (10, 250gy); the PIC for this primer was 0.205 and MI was 0.41 (Table 3).Primer HB-13 revealed five bands ranging in size between 420 to 840 pb; two bands were polymorphic and three band was monomorphic (Fig. 3 and Table 3). The three bands were scored in CI-2 by the dose 10gy, while the highest number of bands (5) was scored in CI-1 by the dose (0,10,250,500gy) of gamma radiation and in CI-2 by the dose of (250,500,750,1000gy) and in CI-3 mutant isolates by the doses of (0,250,500,1000gy); the PIC for this primer was 0.11 and MI was 0.22 (Table 3).Primer HB-15 revealed 6 bands ranging in size between 350 to 960 pb; four bands were polymorphic and two band was monomorphic (Fig. 3 and Table 3). The lowest number of bands four was scored in CI-1 mutant isolate by the dose 250gy and CI-2 by the dose 10gy, while the six bands was scored in all samples except CI-1 by the dose 10,250gy of gamma radiation and CI-2 mutant isolate by the dose of 10gy and the control of CI-3 strain; the PIC for this primer was 0.10 and MI was 0.403 (Table 3).

#### *Cluster analysis*

According to the similarity matrix, (Table 4) Jacquard's similarity index of sample pairs varied between 0.510 and 0.896. Genotypes CI-2(750gy) and CI-3(250gy) with similarity coefficient of 0.896, and genotypes CI-2(10gy) and CI2(1000gy) with similarity coefficient of 0.510 were the most and the least similar genotypes suggesting high level of genetic variability in the strains and mutants, respectively (Fig. 4). The dendrogram obtained from ISSR profiles is represented in Table (4) and Fig. (4) grouped the 18 samples into three major clusters (Fig. 3), placing fourteen accessions from plain region in first cluster, three Mutant Isolate No. CI-1 (1000gy), CI-2 (control and 10gy) in the second cluster and Genotype CI-1(500gy) revealed a completely different banding profile with all 10 selected primers and stood quite distinct from other genotypes in the final cluster. Cluster1 again sub clusters into two clusters placing accessions CI-1(0,10 and 250 gy) and Mutant Isolate No.CI-3(1000gy) in sub cluster 1 and the rest in sub cluster 2. Genetic diversity between different strains can be documented by using different molecular markers (Ali, 2003; Castano and Becerril, 2004). Induced mutations in *Pleutotusostreatus* and notified 10 times higher extra cellular enzyme activities of isolated mutant strains by RAPD- PCR and accounted 64.4 to 93.3% genetic similarities of mutants and wild strains (Lee *et al.*, 2000). ISSR-PCR are perfect as markers for

genetic mapping and population studies because of their abundance and the high degree of polymorphism between individuals within a population of closely related genotypes (Lanham and Brennan, 1998). ISSR markers have been used to study the genetic diversity of some edible mushrooms (Qin *et al.*, 2006; Nazrul and Bian, 2010b). The previously reported annealing temperature for ISSR amplification with different primers (Primmer *et al.*, 2005; Najaphy *et al.*, 2011; Nazrul and Bian, 2011) was not able to amplify clear polymorphic bands, or generated smeared banding patterns.

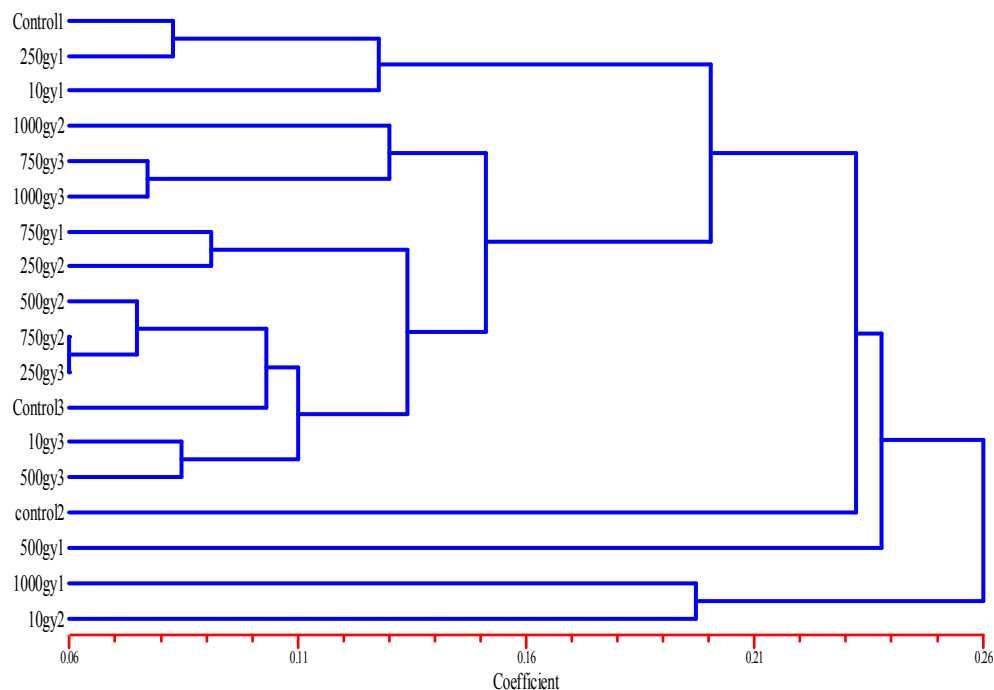


Fig. 4. Dendrogram showing cluster analysis dissimilarity estimates for *Calocybe indica* mutants by gamma irradiation from ISSR

Geno-typed Irradiation dose (gy)	Strain CI-1						Strain CI-2						Strain CI-3					
	0.0	10	250	500	750	1000	0.0	10	250	500	750	1000	0.0	10	250	500	750	1000
0.0	1.00	0.727	0.857	0.633	0.700	0.714	0.659	0.667	0.740	0.706	0.700	0.600	0.708	0.680	0.714	0.729	0.620	0.745
10		1.00	0.854	0.592	0.627	0.608	0.651	0.587	0.604	0.604	0.694	0.660	0.633	0.608	0.708	0.688	0.756	0.818
250			1.00	0.667	0.667	0.680	0.698	0.630	0.673	0.706	0.700	0.667	0.708	0.680	0.787	0.766	0.723	0.822
500				1.00	0.673	0.623	0.596	0.571	0.679	0.712	0.642	0.640	0.680	0.623	0.686	0.667	0.627	0.647
750					1.00	0.685	0.633	0.673	0.843	0.843	0.804	0.706	0.780	0.820	0.784	0.800	0.760	0.712
1000						1.00	0.681	0.688	0.691	0.755	0.685	0.623	0.630	0.698	0.667	0.648	0.642	0.725
0.0							1.00	0.591	0.608	0.640	0.667	0.630	0.604	0.681	0.646	0.625	0.617	0.750
10								1.00	0.714	0.615	0.519	0.510	0.549	0.558	0.528	0.569	0.529	0.612
250									1.00	0.811	0.741	0.679	0.750	0.722	0.722	0.769	0.667	0.717
500										1.00	0.880	0.780	0.820	0.788	0.860	0.804	0.731	0.685
750											1.00	0.813	0.816	0.784	0.896	0.837	0.796	0.745
1000												1.00	0.714	0.755	0.792	0.809	0.844	0.680
0.0													1.00	0.760	0.833	0.851	0.735	0.654
10														1.00	0.800	0.854	0.740	0.760
250															1.00	0.894	0.851	0.760
500																1.00	0.830	0.740
750																	1.00	0.735
1000																		1.00

## Conclusion

In conclusion, the ISSR-PCR analyses showed a medium level of genetic diversity among *C. indica* strains and their mutants. The results of this study indicated that strains of *C. indica* and their mutants show abundant diversity in morphological and genetic diversity, and that ISSR-PCR is a useful tool for selecting potential strains and mutations of mushrooms.

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