Current Research in Environmental & Applied Mycology (Journal of Fungal Biology)
7(3): 144–154 (2017) ISSN 2229-2225
www.creamjournal.org
Article
Doi 10.5943/cream/7/3/1

Wheat flour, an inexpensive medium for *in vitro* cultivation of coprophilous fungus *Coprinopsis cinerea*

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Mohankumar S, Savitha J 2017 – Wheat flour, an inexpensive medium for *in vitro* cultivation of coprophilous fungus *Coprinopsis cinerea*. Current Research in Environmental & Applied Mycology (Journal of Fungal Biology) 7(3): 144–154 (2017), Doi 10.5943/cream/7/3/1

Abstract

Coprinopsis cinerea, a coprophilous basidiomycetous fungus generally called as inky cap mushroom is used as a model organism to study the evolution of fruiting bodies in higher fungi. Herbivorous animal dung is a major source of *Coprinopsis cinerea*, as it contains high carbon, nitrogen, and phosphorus elements. Due to the extensive application of *Coprinopsis cinerea* in Genetics, Molecular Biology and Microbial Biotechnology it is necessary to explore a suitable inexpensive medium for its *in vitro* cultivation. In our present study, we found that 2% wheat flour medium supported the vegetative growth and induced the fruiting body formation within 10 days at 30^{o}C, pH 6 under dark compared to malt extract amended media. The number of fruiting bodies and biomass of fruiting bodies were also found higher in wheat flour medium compared to other media tested with similar cultural conditions

Key words – Basidiomycetes – Biomass – Fruiting body – Growth – Herbivores – dung – Inky cap

Introduction

Since ancient times mushrooms were widely used globally as food for their nutritional and medicinal value (Badalyan 2014). *Coprinopsis cinerea*, a coprophilous basidiomycete, belonging to the family Psathyrellaceae is a model organism for the study of fungal sex due to their short haploid period. Recent studies on *C. cinerea* has shown that it is a reservoir of many bioactive compounds such as antimicrobial peptides (AMPs), insecticidal peptides (Sabotic et al. 2016) and industrially important enzymes (Ruhl et al. 2013)

Copsin, a novel antimicrobial peptide (AMP) isolated from *Coprinopsis cinerea* showed antibacterial activity against several pathogenic bacteria including *Listeria monocytogenes* (Essig et al. 2014). Novel nematotoxic lectins were identified from *C. cinerea* which are toxic against many bacterivorous nematodes including *Aphelenchus avenae* (Plaza et al. 2016). Ethyl acetate extracts of *Coprinus cinereus* (Schaeff) S. Gray s. lat. cultivated on grasses supplemented with cow dung manure exhibited significant activity against *E. coli*, *Candida albicans* and *Aspergillus niger* (Ndyetabura et al. 2010). Cospin, a first fungal trypsin- specific protease inhibitor was identified by Sabotic et al. (2012) from *C. cinerea*, which showed toxicity against fungivorous insects. Methanolic extracts of a species of *Coprinopsis*, *C. atramentaria* revealed antioxidant activity and the compounds were identified as *p*-hydroxybenzoic acid (HA), *p*-coumaric (CoA) and cinnamic acid (CA) (Heleno et al. 2014). Further, Heleno et al. (2014) identified methylated and glucuronated derivatives of phenolic acids of *C. atramentaria*, showed good activity against MCF-

Submitted 27 February 2017, Accepted 9 March 2017, Published 20 July 2017
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7-breastcarcinoma, NCI-H60-non-small cell lung carcinoma, HCT15-colon carcinoma cell lines. It also showed potent antimicrobial and demelanizing activities against *Aspergillus niger, Aspergillus fumigates, Penicillium verrucosum* var. *cyclopium, Listeria monocytogenes, Staphylococcus aureus and Bacillus cereus* etc. *C. cinerea* also possess enzymes with novel characteristics (Kaur et al. 2011)

Beyond any doubt, herbivorous animal dung is a rich substratum for the growth and sporulation of coprophilous fungi, especially *Coprinopsis cinerea.* But, however, due to hygienic point of view, laboratory cultivation in dung or dung extract media are not recommended. Therefore, there is an immediate need to search for better and cheaper medium for vegetative growth, fructification and biomass production of *Coprinopsis cinerea*, which possess several significant metabolites of commercial value. Several reports are however, available on the growth of *Coprinopsis cinerea* on synthetic media and malt extract supplemented media is reported to be the best among them. The present study was aimed to identify a medium for faster growth, better biomass and fruit body production of *Coprinopsis cinerea* under laboratory conditions.

Materials & Methods

Isolation of *Coprinopsis cinerea*

Coprinopsis cinerea was isolated from fresh dung samples of various herbivorous animals such as cow, horse and buffalo. The samples were collected in a zip lock polythene bag from Hessarghatta, Bangalore during rainy season. The dung samples were brought to the laboratory and aseptically transferred to a sterilized flask (Richardson 2001). The flasks were incubated at room temperature (28°C) for 25 days for the formation of fruiting bodies. The fruiting bodies from each sample were removed aseptically and stored at -20°C for further use

Molecular Characterization of the isolates

The fungus was grown on malt extract agar medium and the mycelial mat was scrapped from the agar plates and used for genomic DNA isolation using Qiagen DNeasy Kit. The DNA was amplified by PCR amplification experiments using ITS primers with the forward sequence ITS1 (5´-TCCGTAGGTGAACCTGCGG-3´) and reverse sequence ITS4 (5´-TCCTCCGCTTATTGATATGC-3´) (White et al. 1990). The amplified products were purified using geneO-spin PCR product purification kit (geneOmbio technologies Pune; India) and sequenced using an ABI PRISM Bigdye terminator V3.1 kit (Applied Biosystems USA). The sequences were analyzed using Sequencing Analysis 5.2 software. The sequence similarity was detected by Blastn (Altschul et al. 1997) against the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/). The sequences derived from these studies have been deposited in NCBI GenBank (Benson et al. 1999). Multiple sequence alignments were performed using Clustal W (Thompson et al. 2002). The evolutionary distance was calculated by the method of Jukes and Canter (1969). Phylogenetic tree was constructed by the neighbor-joining method and tree topologies were evaluated by bootstrap analysis using MEGA6 (Molecular Evolutionary Genetic Analysis) (Tamura 2013).

In vitro cultivation of *Coprinopsis cinerea*

The fruiting bodies of *C. cinerea* grown on cow, horse and buffalo dung were collected and washed thoroughly with distilled water (twice) and chopped into small pieces of approximately 1cm length using sterile scalpel and surface sterilized using sodium hypochlorite (4%), ethanol (70%) and sterile distilled water. The small pieces of sterilized fruiting bodies were inoculated on different agar media: Malt extract agar (MEA; 3% malt extract, 0.5% mycological peptone, 2% agar); Malt extract- yeast extract agar (MYA; 1% malt extract, 0.2% yeast extract, 2% agar); Glucose-peptone agar (GPA; 4% glucose, 1% peptone, 2% agar); Yeast extract-peptone agar (YPA; 1% yeast extract, 2% bactopeptone, 2% agar); Yeast extract malt extract glucose/ Tryptophan agar (YMG/T; 0.4% yeast extract, 1% malt extract, 0.4% glucose, 0.01% tryptophan, 2% agar); Malt extract yeast extract casamino acid agar (MYC; 1% malt extract, 0.2% yeast

extract, 0.2% casamino acid, 2% agar); Dung extract agar (DEA; 0.2% Dung extract, 2% agar); mKjalke agar (1% yeast extract, 2% glucose, 0.05% CaCl2 2H2O, 0.02% KH2PO4, 5 mg MgSO4, 2% agar); Potato dextrose agar (PDA; 3.9% potato dextrose agar); YMG agar (0.4% yeast extract, 1% malt extract, 0.4% glucose, 2% agar) and the plates were incubated for 7 days at 30°C, pH 6 under dark conditions (Ruhl et al 2013). After the incubation period the plates were observed for morphological characteristics of the colonies.

Cultivation of *Coprinopsis cinerea* on wheat flour medium

Wheat flour medium was prepared with varying concentrations of wheat flour (1%, 2%, 3% of wheat flour for wheat flour broth and with addition of 1.5% agar for wheat flour agar) and inoculated with 5mm mycelial disc of *Coprinopsis cinerea* species for 7days for radial growth, 10 days for biomass production and 15 days for fruiting body production at 30°C, pH 6 under dark conditions. The colony diameter was measured at regular intervals and the numbers of fruiting bodies were also counted. Fresh and dry weight of the biomass was also noted as given above.

Statistical Analysis

All the experiments were done in triplicates and the mean values, standard deviation and standard error were calculated using the software IBM SPSS Statistics for Windows, Version 20.0 (Armonk, NY: IBM Corp). The results were expressed as mean ± standard error.

Results

Identification of *Coprinopsis* spp.

Three fruiting bodies were isolated from three different dung samples and designated as C1, C2 and C3. The length of the stipe (Figs. 2A, 2B) and diameter of the pileus was measured (Table 1). The spore prints (Fig. 1) were dark brown and the basidiospores were black, ellipsoidal with a rounded apex and a pointy central germ pore (Fig. 2C). Based on these morphological data the fruiting bodies were identified as *Coprinopsis cinerea.*

Table 1 Morphological identification of *Coprinopsis cinerea* isolated from different dung samples.

Source of the dung	Codeofthe Isolate *	Length of the stipe (cm)*	Width of the pileus(cm)*	Diameter of the spore (µm)*
Cow	C1	8	1.5	8
Horse	C2	12	2.8	9
Buffalo	C3	11	2.3	8

Molecular characterization of the isolates

The molecular identification of the three isolates (C1, C2 and C3) was done by sequencing and characterizing the gene encoding for 18S rRNA. The sequence was queried in nucleotide BLAST search from NCBI to find the homology with the existing species of *Coprinopsis*. The first nucleotide sequence C1 isolated from cow dung was 389 bp, the second nucleotide sequence C2 isolated from horse dung was 620 bp and the third nucleotide sequence C3 isolated from buffalo dung was 466 bp. The nucleotide sequence showed 98% (C1) and 100% (C2 and C3) identity with *Coprinopsis cinerea*. The three nucleotide sequence data were deposited to GenBank and obtained the accession numbers *Coprinopsis cinerea* strain C1 (KX468976), *Coprinopsis cinerea* strain C2 (KX468976) and *Coprinopsis cinerea* strain C3 (KX468977). The phylogenetic trees were constructed for all the three isolates by obtaining the ITS sequence of closely related taxa (Fig. 3).

In vitro cultivation of *Coprinopsis cinerea* and selection of a suitable strain for further studies

The radial growth of the fungal colony on different media was measured for all the three strains of *Coprinopsis cinerea* (Fig. 4). Among all the media tested, successful growth was observed in only six media *viz* MEA, MYA, YMGTA, MYCA, YMGA, WFA and no growth was

found in GPA, YPA, DEA, PDA and mKjalke media. Interestingly, we observed luxuriant and faster growth of *C. cinerea* strain C2 in MEA, YMGTA and WFA compared to other two strains of *C. cinerea* (C1 and C3) incubated for 7 days at 30° C, pH 6 under dark condition (best suited cultural condition for the cultivation of *C. cinerea in* laboratory). Based upon these results we selected *C. cinerea* strain C2 for further studies.

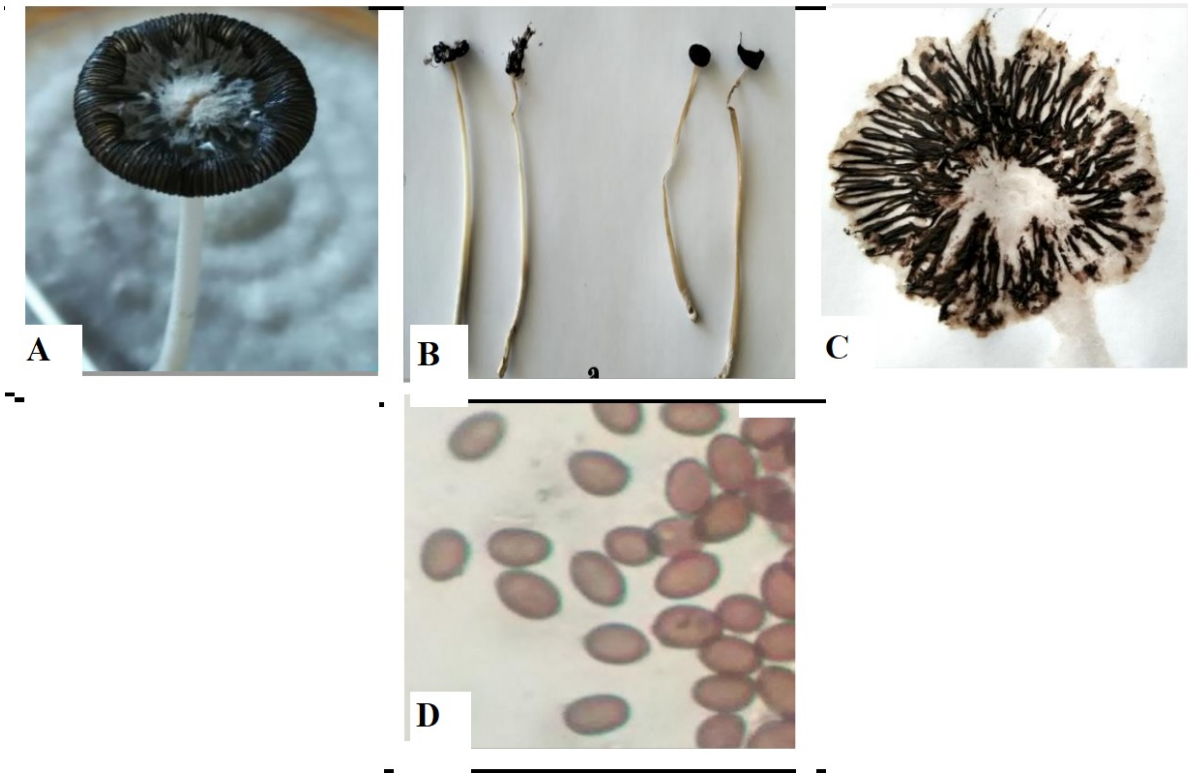

Fig. 1 – Fruiting bodies (A, B), spore print (C) and spores (D) of *Coprinopsis cinerea*

Effect of solid and liquid media on *Coprinopsis cinerea* strain C2 growth

Coprinopsis cinerea strain C2 isolated from horse dung has showed growth in all the six media but significant growth was observed in wheat flour agar (WFA) medium (Fig. 4). Average radial growth of 9cm was achieved within 7 days of incubation in WFA compared to other media at 30° C, pH 6 under dark, whereas poor growth of 3cm and 5cm was observed in YMGA and MYA media respectively, incubated under similar cultural conditions. We have also observed production of fresh (84.96 grams/l) and dry (7.9grams/l) biomass was higher in WFB compared to other liquid media (Fig. 5) and the least fresh (2.2g/l) and dry (24.46g/l) biomass production was found in YMG media.'

concentrations (1 and 3%) (Figs. 5, 6). The maximum biomass (fresh, 85.22g/l and dry 7.7g/l) production was obtained at 2% wheat flour concentration compared to other two concentrations were tested (Fig. 7).

We also tested the suitable concentration of wheat flour for the growth of *C. cinerea* strain C2. Medium with 2% concentration of wheat flour showed faster radial growth of 9cm in solid medium within 7 days of incubation at 30° C, pH 6 under dark compared to other two

Discussion

Coprinopsis cinerea is commonly known as gray shag or inky cap mushroom due to its black colored pileus and deliquescence or autolysing property. It is particularly a suited organism to study meiosis in living system due to its synchronous meiotic development and prolonged prophase (Burns et al. 2010). It is also a model organism for studying fungal sex and evolution of fruiting bodies in fungi. *C. cinerea* is a common flora of herbivores dung because of its rich organic matter.

Due to hygienic concern of the dung and extensive application of *C. cinerea* in various fields, the present study was aimed at developing an inexpensive, hygienic medium suited for routine use for cultivation.

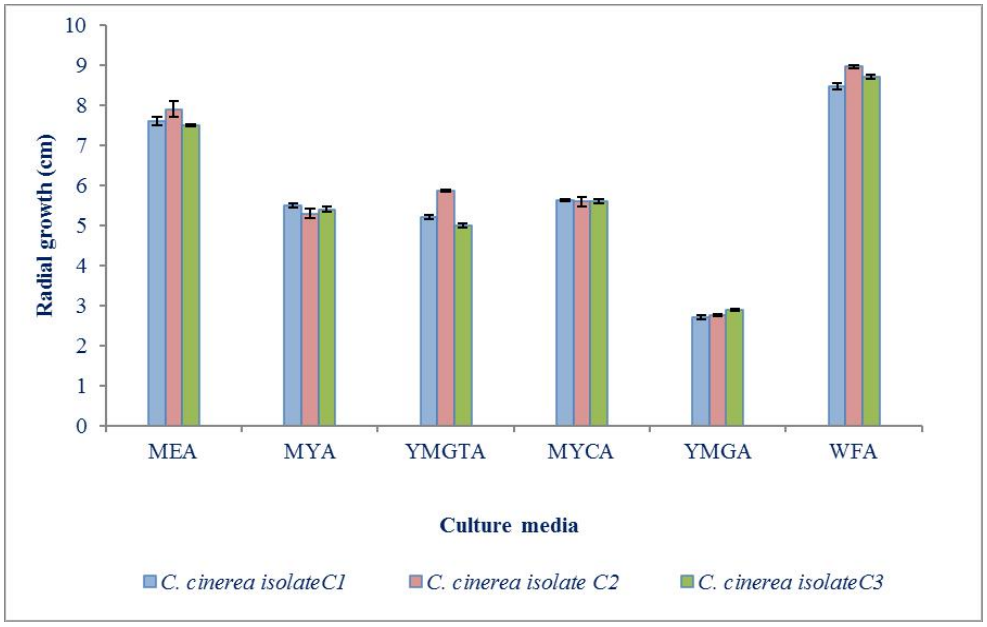

Fig. 2 – Phylogenetic tree showing relationships of *Coprinopsis cinerea* strain C1, C2, C3 and related species of *Coprinopsis* identified on the basis of 18S rRNA gene sequences, using neighbor joining method in MEGA 6. Bootstrap values are shown at the branch points.

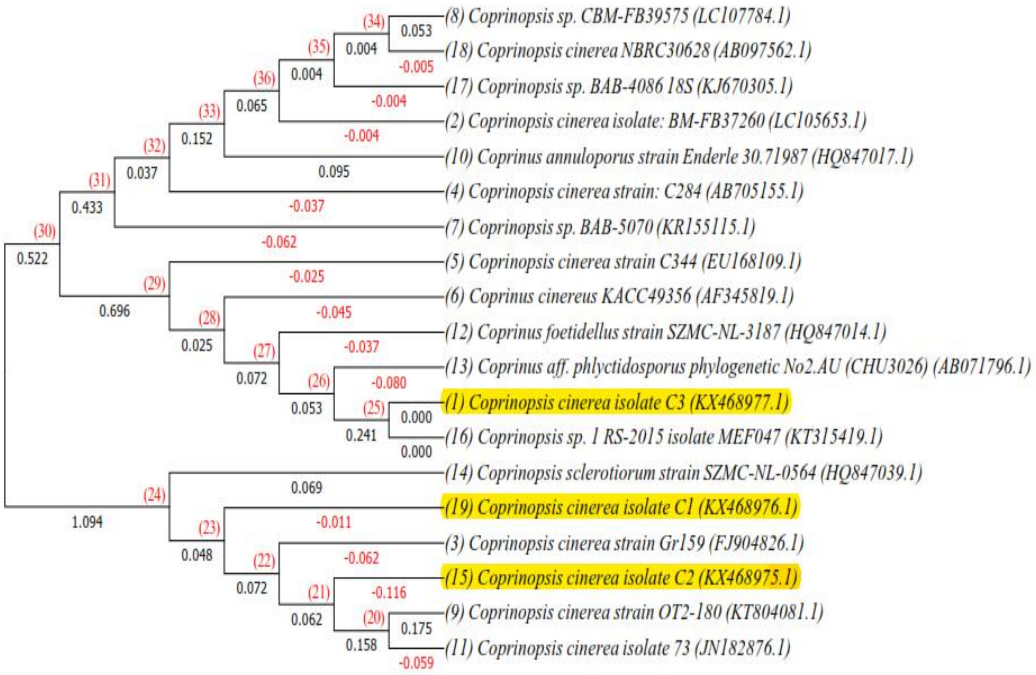

Fig. 3 – Growth of *Coprinopsis cinerea* strain C1, C2 and C3 on different culture media incubated for 7 days at 30° C, pH 6 under dark: MEA- Malt extract agar, MYA- Malt extract yeast extract agar, YMGTA- Yeast extract malt extract glucose tryptophan agar, MYC-Malt extract yeast extract casamino acid agar, YMGA-Yeast extract malt extract glucose agar and WFA- Wheat flour agar.

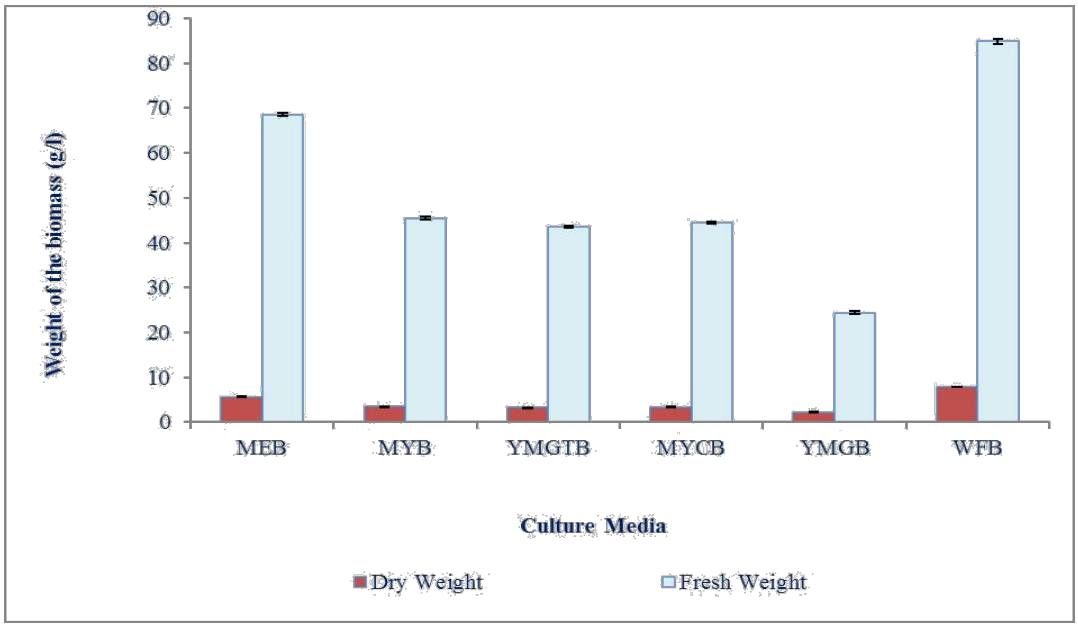

Fig. 4 – Biomass production of *Coprinopsis cinerea* strain C2 on different culture media incubated for 10 days at 30° C, pH 6 under dark: MEA- Malt extract agar, MYA- Malt extract yeast extract agar, YMGTA- Yeast extract malt extract glucose tryptophan agar, MEA- Malt extract yeast extract casamino acid agar, YMGA-Yeast extract malt extract glucose agar and WFA- Wheat flour agar.Six types of media induced fruiting body production of *C. cinerea* strain C2 incubated for 15 days at 37° C, pH 6 (Fig 8). However, wheat flour agar medium amended with 2% wheat flour supported faster growth and induced more number (4) of fruiting bodies compared to other media. In MYC medium we have observed only one fruiting body formation.

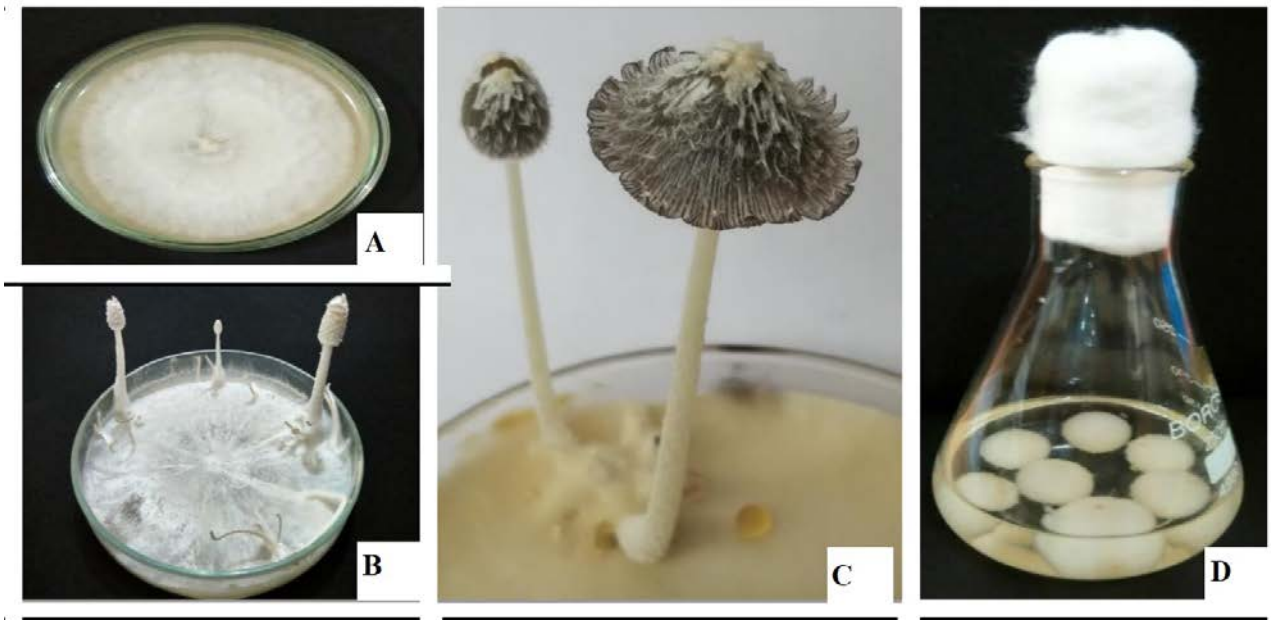

Fig. 5 – Growth of *Coprinopsis cinerea* strain C2 on 2% wheat flour medium incubated for 10 days at 30° C, pH 6 under dark: A) Radial growth B) Fruiting bodies C) Mature fruit body D) Growth on wheat flour broth.

Coprophilous fungi are generally cultured on horse dung extract medium. But, however, the extract is not effective for induction of more number of fruiting bodies as it depends on the freshness of the dung (Lange 1952). Cultures grown from stored dung failed to initiate fruiting body production although they supported normal vegetative growth (Eilers, 1972).

Media rich in disaccharides play a major role in mushroom growth and fruiting body induction of mushrooms compared to monosaccharide (Zhou et al. 2016: Cosgrove 1997). The nutrient profile of mushrooms influenced by the type of substrate on which they are cultivated (Kulshreshtha et al. 2014). Therefore, in our study, we searched for a better substrate providing rich source of disaccharides and other nutrients for the cultivation of *C. cinerea* under laboratory condition.

Several researchers have used synthetic media for growth and biomass production (Essig et al. 2014: Kaur et al. 2011: Dulay et al. 2016) of *C. cinerea*. Cultivation in combination with yeast extract, malt extract and peptone amended media (Sabotic et al. 2012) are although common, they failed to induce fruiting body and maximum biomass production. According to Jaekel et al. (2012) wheat flour has better nutritional and functional values, as it is a rich source of carbohydrates (80.76%), proteins (15.41%), lipids (2.12%) and ash (1.71%) and also contains fibers, minerals, vitamins, phenolic compounds, phytic acid, and phytoestrogens among other nutrients (Kulawinek et al. 2008). Carbohydrates in wheat flour are a mixture of disaccharide as well as monosaccharide such as sucrose (2.16 mg/g), maltose (0.57 mg/g), fructose (0.53 mg/g), and glucose (0.45 mg/g) (Codina et al. 2013). We observed, the biomass and number of fruiting bodies increased significantly in wheat flour medium compared to malt extract amended media. Moreover, the wheat flour required for the growth of *Coprinopsis cinerea* was observed to be very low, just 2%, which enhanced the maximum growth and production of fruiting bodies.

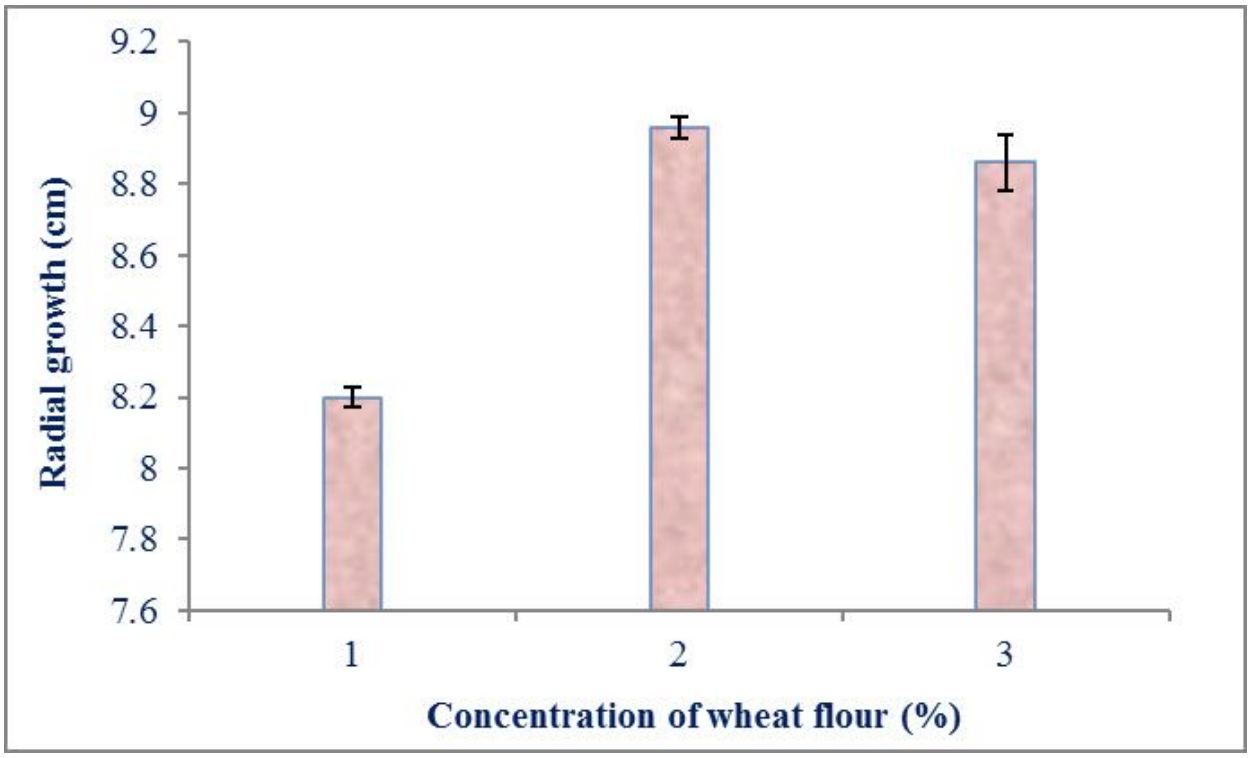

Fig. 6 – Growth of *Coprinopsis cinerea* strain C2 on different concentrations of wheat flour media incubated for 7 days at 30° C, pH 6 under dark.

Our present study reports for the first time that wheat flour (2%) provides complete nutrient for maximum production of biomass and induction of fruiting bodies in *C. cinerea* without any additional supplement compared to other media reported earlier and tested by us. The preliminary work reported in this paper would certainly benefit the researchers working on mushrooms in general, and *C. cinerea,* in particular, as they have tremendous potential value in pharmaceutical and food industries.

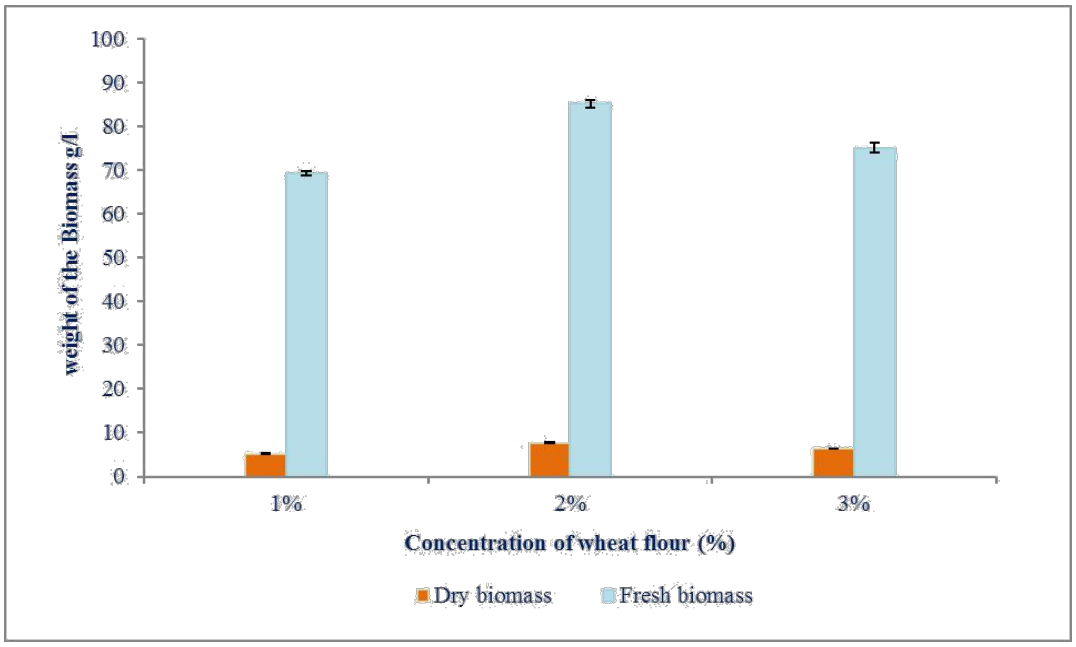

Fig. 7 – Biomass production of *Coprinopsis cinerea* strain C2 on different concentrations of wheat flour broth incubated for 10 days at 30° C, pH 6 under dark

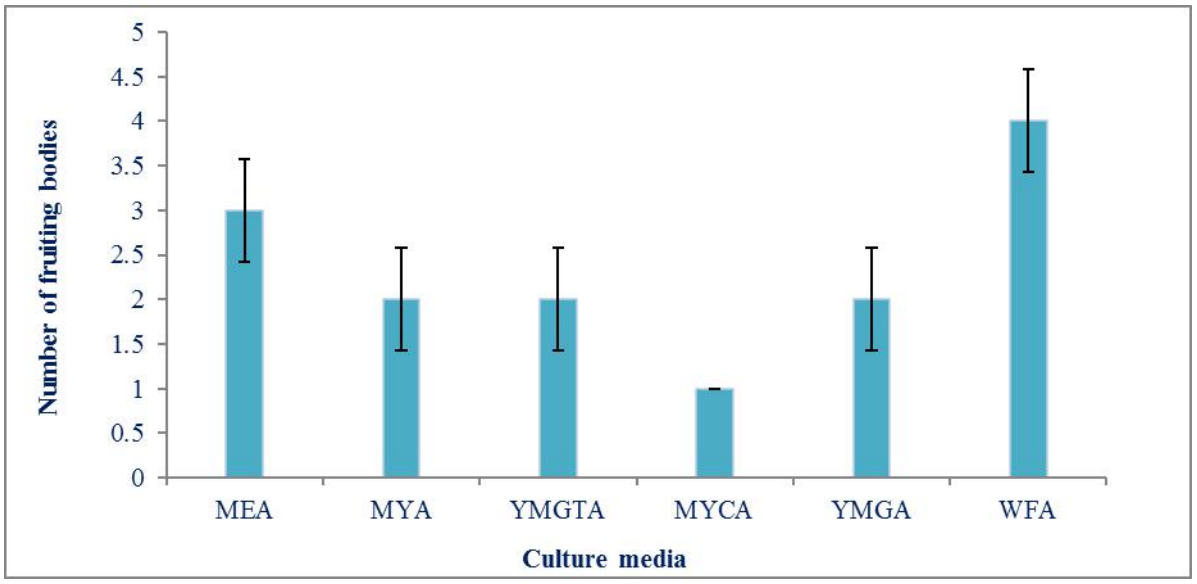

Fig. 8 – Fruiting body production of *Coprinopsis cinerea* strain C2 on different culture media incubated for 15 days at 30° C, pH 6 under dark: MEA- Malt extract agar, MYA- Malt extract yeast extract agar, YMGTA- Yeast extract malt extract glucose tryptophan agar, MYCA- Malt extract yeast extract casamino acid agar, YMGA-Yeast extract malt extract glucose agar and WFA- Wheat flour agar

Acknowledgements

The authors would like to thank the Department of Microbiology and Biotechnology, Bangalore University, Bangalore for providing the facility to carry out this research work.

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