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Cultivation of wild indigenous *Agaricus bisporus* and *Agaricus subrufescens* from Pakistan

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Abstract

Cultivation potential of two wild indigenous species i.e. *Agaricus bisporus* (W-01) and *A. subrufescens* (KH 407) was estimated. Species were collected from Khyber Pakhtunkhwa region of Pakistan. Mycelial growth was obtained at Compost Extract Agar medium, proceeded for spawn production using wheat and sorghum grains. Wheat straw based mushroom compost supplemented with chicken manure, urea and gypsum was prepared for spawning. Temperature optimization was done at two temperatures of 20℃ and 28℃. Spawn run rate was maximum for both species at low temperature. *Agaricus subrufescens* (KH 407) is characterized by crystal like exudates in its cultures. Cultivation trails were successful yielding large fruit bodies of *A. bisporus* (W-01) and primordial formation in *A. subrufescens* (KH 407). Production rate of fruiting bodies was higher *in A. bisporus* (W-01) as compared to *A. subrufescens* (KH 407).

Key words – Casing – native species – Pin heads – Spawn run rate – Temperature optimization

Introduction

Genus *Agaricus* L. is terrestrial, saprophytic fungus, containing both edible and poisonous taxa, found growing on soil in forest floors, lawns, fields, manure piles and wood logs often called as “meadow mushrooms” (He et al. 2018). Records from world represent more than 500 species of genus *Agaricus* (Chen et al. 2019, Zhao 2020). Recent infra generic investigations divided this genus into further six subgenera and about 24 sections (Zhao et al. 2016, Chen et al. 2017, Chen et al. 2019). Amongst these sections *A.* sect. *Biverales* and *A.* sect. *Arvenses* mainly contain many edible species while section *Xanthodermatei* consists of all the toxic species of genus *Agaricus* (Kerrigan 2016).

Many species of genus *Agaricus* viz. *A. campestris* L., *A. bisporus* (J.E. Lange) Imbach and *A. bitorquis* (Quel.) Sacc. are common edible mushrooms. *Agaricus bisporus* is widely cultivated mushroom in about 70 countries throughout the world (Kuo 2018). Fruiting bodies are generally characterized by pilei that are not brightly colored, mostly pink or light to dark brown lamellae and partial veil or its remnants often present on the stipe. These are widely cultivated for food as well as a medicinal resource (Kerrigan 2016).

Agaricus bisporus belonging to *A.* sect. *Biverales* also known as white button mushroom which is considered as a good edible, highly nutritious and, widely consumed mushroom (Ayyub et al. 2014, Kerrigan 2016). *Agaricus bisporus* was first cultivated in France in 1600 (De Leon 2003). French botanist Joseph Pitton de Tournefort in 1707, for the first time, provided the description for commercial cultivation of *Agaricus bisporus* (Spencer 1985).

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Agaricus subrufescens Peck. is an edible species, also called as "Almond mushroom" due to its almond like aroma and taste (Kerrigan 2005). *Agaricus blazei* is a synonym used previously for this species (Zied et al. 2012). It was described from USA New York in 1893 belongs to *Agaricus* section *Arvenses* (Wisitrassameewong et al. 2012). *Agaricus subrufescens* is significant due to its therapeutic, and nutritional properties which has been strongly demonstrated in about 1000 clinical articles (Velázquez-Narváez et al. 2018). This species is recorded from Asia, South America, Europe so far (Chen et al. 2016) and now from Pakistan.

In Pakistan, *A. bisporus* is widely cultivated in Margala Mushroom farm, Islamabad on industrial level while, at smaller scale some home based setups provide supplies. According to an estimation total small scale mushroom production in Pakistan was of 155.6 kg in 2010 (Tahir & Hassan 2013). During last 35 years, an increase in world mushroom production from about 1 billion kg to 34 billion kg has been reported that is more than 30 folds as compared to the previous records (Royse et al. 2017). Our objectives were to analyze the cultivation potential of indigenous *Agaricus* spp. and to prepare culture bank of important *Agaricus* varieties that will help to produce strains with better characteristics and temperature tolerance in future.

Materials & methods

Sampling and procurement of mycelial culture

Agaricus specimens were collected during moonson season from Peshawar and Khanspur of Khyber Pakhtunkhwa province, Pakistan. Basidiocarps were washed with autoclaved tap water and preserved at –20℃. Wheat straw (1kg) was boiled in tap water (5 liters) for 30 minutes and filtered to prepare compost extract, which was supplemented with 10 g glucose and 20 g agar to prepare compost extract agar (CEA) medium. Cultures were obtained using CEA medium with pileal tissues and gills of basidiocarps as inoculum. Cultures were maintained on media plates through subculturing and strains were developed for both *Agaricus* species viz. *A. bisporus* (W-01) and *A. subrufescens* (KH 407).

Preparation of spawn

The spawn was prepared by using wheat (*Triticum aestivum*) and sorghum (*Sorghum bicolor*) grains (250 g for each sample). Grains were washed and then soaked in water for 24 hours for maximum absorption of water. Grains were boiled for 30 minutes and excessive moisture was removed using blotting paper. Three replicates of spawn with wheat and sorghum grains were separately prepared. The jars were sterilized and grains were supplemented with 4% of gypsum ($CaSO_4$) and 2% of lime ($CaOH_2$). Finally, mycelium of pure culture strains of *Agaricus bisporus* (W-01) and *A. subrufescens* (KH 407) was added in both spawn media. Mycelial disc (9 mm) of pure culture per jar were used as an inoculum for both of the *Agaricus* species. Sealed jars were incubated at 25ºC for 20 days (Ishaq et al. 2017).

Compost preparation for mushroom cultivation

Wheat straw was purchased from local market of Lahore. Substrate was watered for three consecutive days to moist it thoroughly. On third day, supplements were added i.e. urea and chicken manure in the substrate and all the ingredients were put together to form a uniform mixture. Initial pile of about 120 cm was made. The pile was thoroughly mixed four times during this period. Approximately, 1 kg of gypsum was added after third turning and remaining after fourth. After 21 days the color of substrate changed to dark brown, temperature of pile reached about 75℃ due to microbial activity and the fermented material gave ammonia like odor. The prepared compost was then transferred to polypropylene bags and tightly closed with rubber bands. Double sterilization was done in autoclave at 121℃ and 15 lb/inch^2 for 15 – 20 minutes to ensure quality of compost before utilization (Kumar et al. 2017).

Spawning and spawn running

For spawning 25 g of prepared spawn was used to inoculate per kg of compost. Spawn was placed in layers inside the compost bag within the center of substrate and again covered with more compost material under sterilized conditions. After bedding the compost bags were sealed and kept at 25℃ in an incubator. Spawn run rate/mycelial extension was also measured.

Temperature optimization

Temperature optimization was done at 20℃ and 28℃ for both species respectively. Test tubes were monitored with intervals of 48 hours for mycelial extension.

Casing

Casing was done after complete mycelial colonization of compost bags. Casing layer sterilized peat moss mixed with 4% lime along with soil mixture. About 4 cm layer was added for fruiting in spawn run compost.

Fleshing/cropping

After 20–25 days, as the mycelium completely colonized the compost bags, holes were made in the compost bags and some autoclaved water was sprinkled over the bags in order to maintain moisture content at optimum level.

Primordial formation and harvesting

Pin heads or primordial appearance was monitored while maintaining humidity and temperature requirements. Mushrooms were picked gently using a knife or cutter from the compost bags after 7–12 days of primordial formation and stored in sterilized bottles.

Results

***Agaricus bisporus* (J.E. Lange) Imbach W-01**

Morphological features – Basidiocarp: The color of fruit body is white to creamy with few grey to brown scales on pileus, pileus measuring 4.5–5.5 cm in diameter. Odor and taste, mild and meaty. Faint brown discoloration upon bruising.

Colony morphology – After 21 days colony diameter reached up to 3.3 cm on CEA medium at 28℃. Colony was surface to aerial with circular shape and white color thread like mycelial growth in concentric rings, Colony border was clear, white and cottony in texture. Shown in Fig.1

Material Examined – Pakistan KPK, Buner, at 688 m a.s.l., solitary to gregarious on soil, 30 July, 2017, Hira Wahab, W-01 (LAH 35849).

***Agaricus subrufescens* Peck KH407**

Morphological features – The color of pileus surface is reddish brown, pileus measuring up to 7.5 cm in height. Odor and taste, almond-like. Faint reddish-brown discoloration upon bruising.

Colony morphology – Colony was whitish grey with dense felt of hyphae somewhat fragmented and irregular with determined margins that were raised from media surface. Size was 2 cm – 5.5 cm in diameter after 7–21 days of inoculation. Aerial growth was slow with crystal grey exudates. Odor was fruity. Margins were white and hyphal filiform. Texture was fluffy. (Fig. 2)

Material Examined – Pakistan KPK, Hazara, Khanspur, Mukshpuri track, at 2250 m a.s.l., gregarious on rich grounds, 25 July, 2017, Hira Bashir, KH407 (LAH 35853).

Spawn production and spawn running of *Agaricus* species

The mycelium of *Agaricus* species differs in colonization rates from each other on two types of grains used for spawn production (Fig. 3). On wheat grain, spawn production was maximum for *A. bisporus* W-01 and moderate for *A. subrufescens* (KH 407).

Spawn efficiency showed potential strains with maximum mycelial colonization. All strains of *Agaricus bisporus* W-01 and *A. subrufescens* (KH 407) were efficient for spawn production on sorghum grains. Strains varied in efficiency and spawn running depending upon the quality of mycelial growth as shown in Table 1 and Fig. 4.

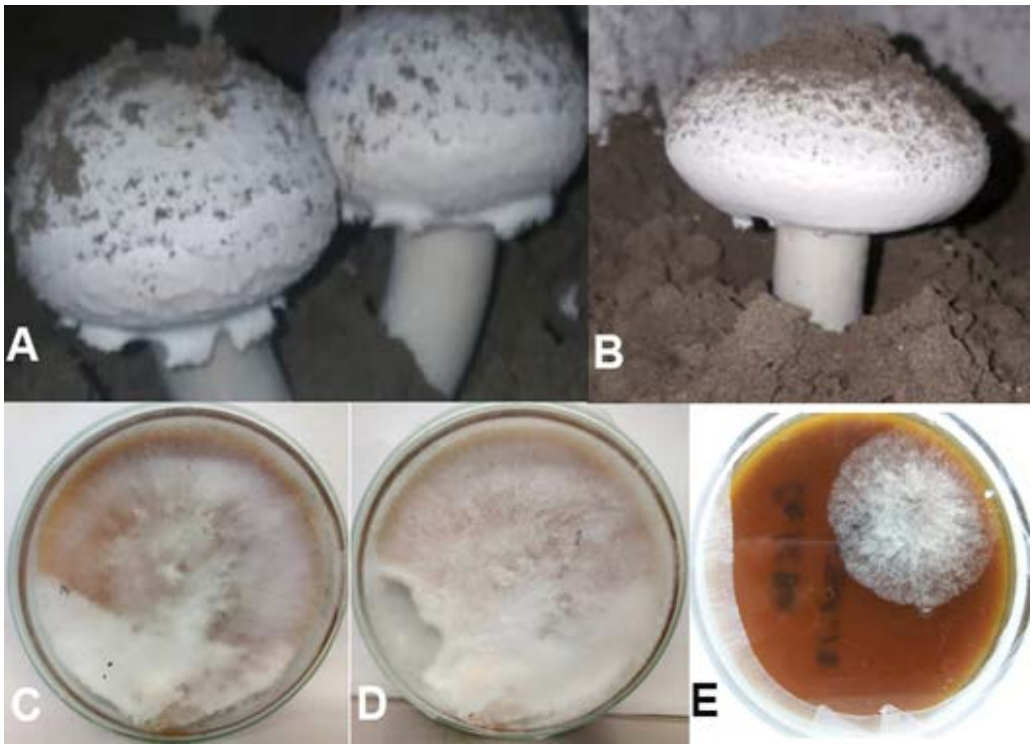

Fig. 1 – Fruiting bodies and cultures of *Agaricus bisporus* (W-01)

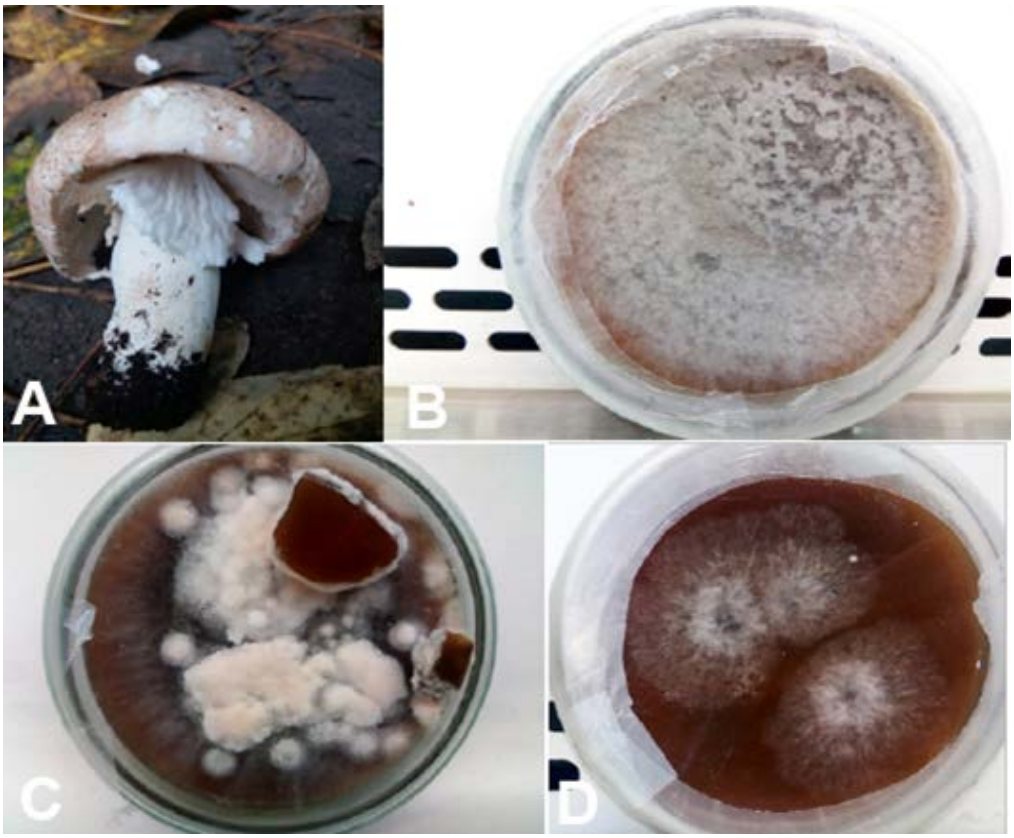

Fig. 2 – Fruiting bodies and cultures of *Agaricus subrufescens* KH407

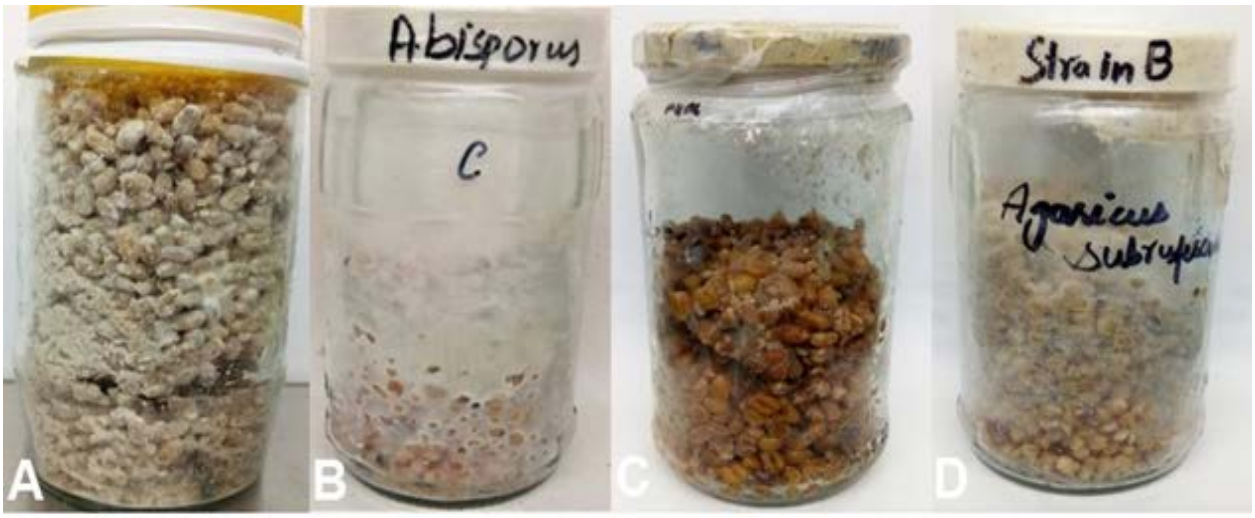

Fig. 3 – Spawn of *Agaricus bisporus* and *Agaricus subrufescens* (KH 407) on wheat and sorghum grains

Table 1 Spawn Efficiency of *Agaricus bisporus* (W-01) and *Agaricus subrufescens* (KH 407)

***Agaricus* Strains**		**Mycelial colonization on Sorghum grains (mm/ 25 days)**	**Spawn run rate = Mycelial colonization on compost (mm/ 30 days)**
Agaricus bisporus (W-01)	*A*	67	75
	B	52	70
	C	78	145
Agaricus subrufescens (KH 407)	*A*	32	75
	B	65	124
	C	50	135

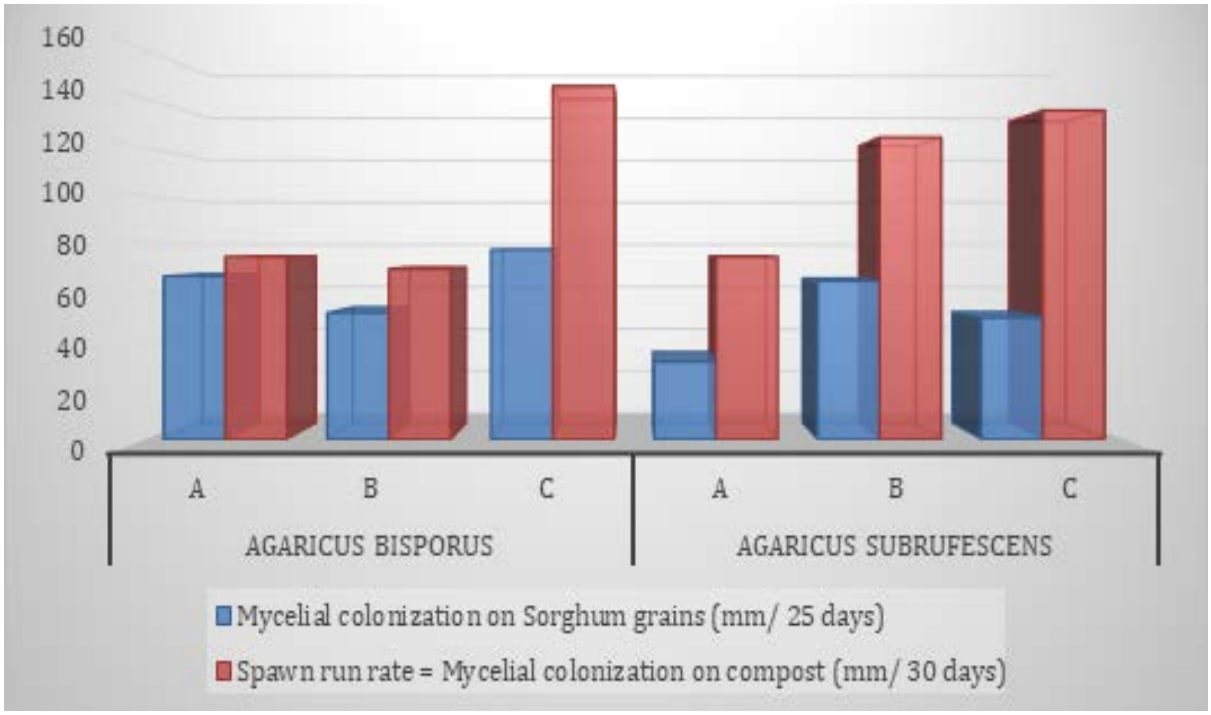

Fig. 4 – Spawn run rate and spawn efficiency for *Agaricus bisporus* (W-01) and *Agaricus subrufescens* (KH 407)

Temperature Optimization

Temperature optimization was also done at two different temperatures (20℃ and 28℃) for all the developed strains (Fig. 5). It was found that 20℃ was more suitable for growth of both species. Findings showed that *A. bisporus* W-01 strains had maximum growth at 25℃. Mycelial growth of *A. subrufescens* (KH 407) was stimulated at a lower temperature of 20℃.

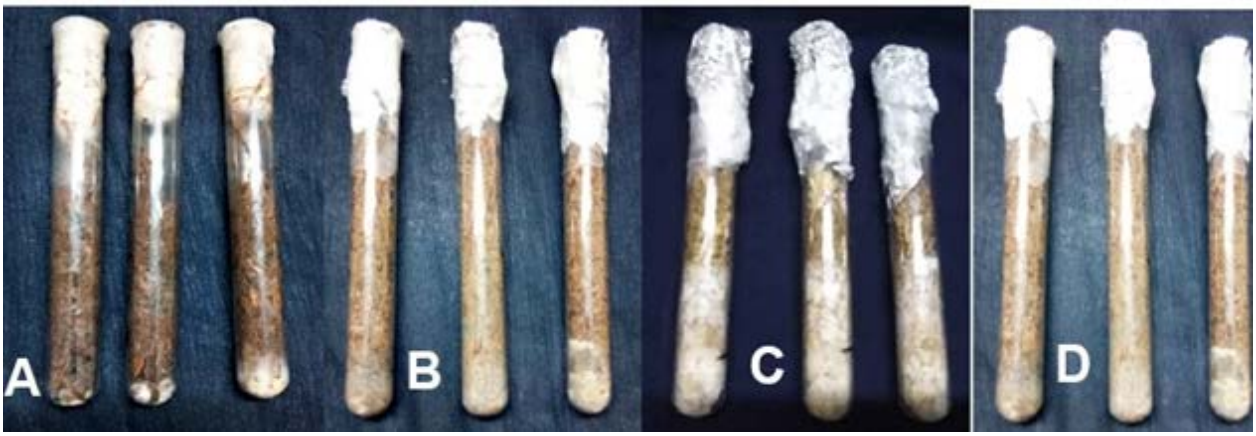

Fig. 5 – A, B Linear Growth of *Agaricus bisporus* (W-01) at 28℃ and 20℃. C, D Linear Growth of *Agaricus subrufescens* (KH 407) at 20℃ and 28℃.

Cultivation Potential of *A. bisporus* W-01 and *A. subrufescens* (KH 407)

Complete mycelial colonization was observed in spawn run compost after 12th day of peat and soil casing. Pin heads of *A. bisporus* W-01 emerged within 7–10 days. Maturation period for fruiting bodies of *A. bisporus* W-01 was of 5–7 days after pin head formation (Fig. 6). *Agaricus subrufescens* (KH 407) took 55–60 days for primordial formation (Fig. 7). Pin heads of *A. bisporus* W-01 appeared after 7–10 days of casing application at 20℃ and maximum development of basidiocarp was obtained in 5–7 days. Primordial formation was observed for *A. subrfescens* (KH 407) in 2 months after peat moss casing.

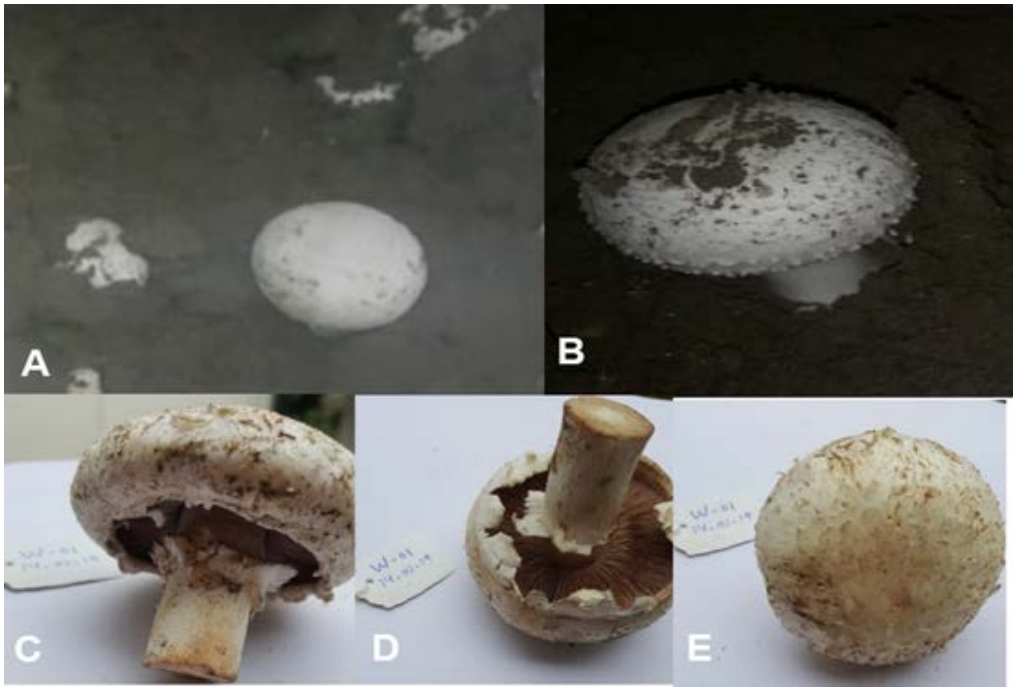

Fig. 6 – Cultivation of *Agaricus bisporus*. (W-01). A Pin head emergence. B Fruiting body of *Agaricus bisporus* (W-01). C Harvested Basidiocarp. D Gills and stipe view. E Pileus view.

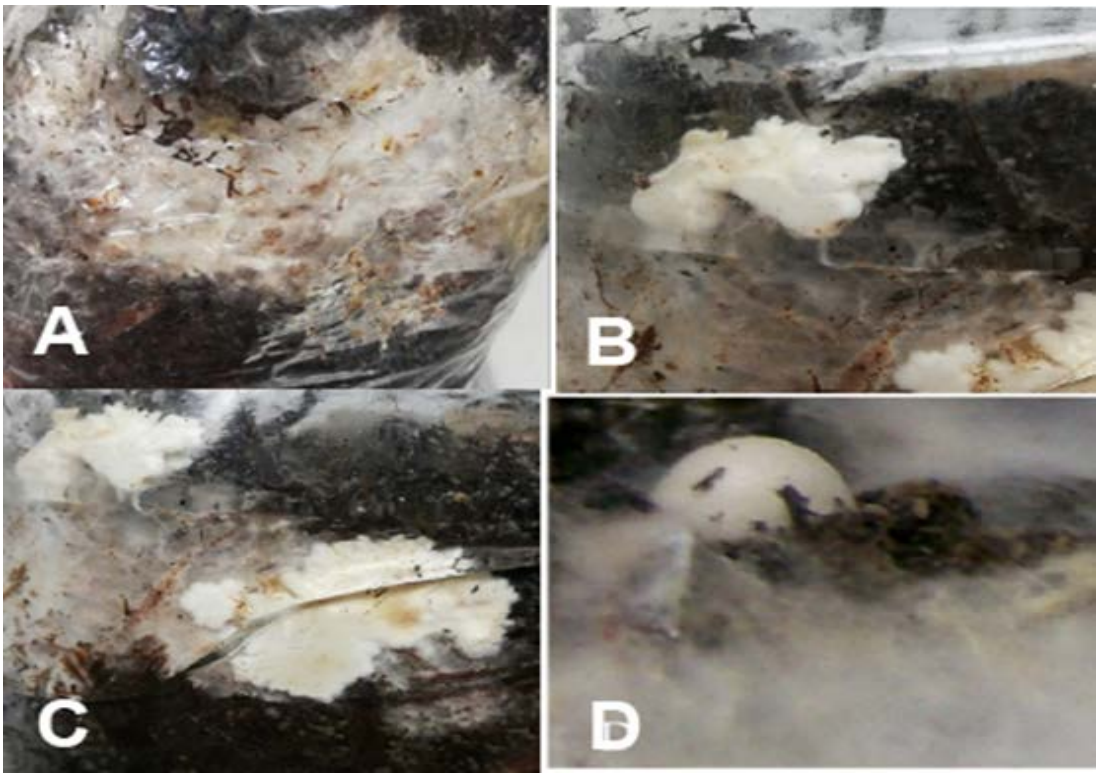

Fig. 7 – Cultivation of *Agaricus subrufescens* (KH 407). A Spawn run composts mycelial growth of *A. subrufescens* (KH 407). B Dense mycelial aggregation. C Initial primordial development. D Pin head emergence of *A. subrufescens* (KH 407).

Discussion

In this study, *Agaricus bisporus* W-01 and *A. subrufescens* (KH 407) showed maximum growth on CEA medium. Similar work has been done by Salmones et al. (2018) and they used malt extract, yeast extract and compost extract media for culturing of *Agaricus bisporus* and the results showed highest growth on compost extract medium. Cultures of *A. subrufescens* (KH 407) were fragmented with crystal grey exudates in our strains similar to the findings given by Hamedi et al. (2012). During this study, the spawn of *A. bisporus,* W-01 and *A. subrufescens* (KH 407) were prepared on wheat as well on sorghum grains. Previous practice of spawn production for *A. bisporus* is on wheat grains (Albertó 1997). It was observed that mycelia of all the species vigorously colonized sorghum grains. *Agaricus bisporus* W-01 showed effective colonization on both grains used for spawn production. Sorghum grains provide the mycelium with more respiration, as there are many pores in sorghum as compared to wheat grains. Due to small size sorghum grains are more compact and have large surface area thus, facilitating better mycelial colonization (Narh et al. 2011). Temperature optimization was also done at two different temperatures for all the strains developed. It was found that 20℃ was suitable for growth of *A. bisporus* W-01 and *A. subrufescens* (KH 407). Ali et al. (2015) also investigated temperature requirements for growth of *A. bisporus* and *A. bitorquis* strains. Their findings showed that *A. bisporus* strains had maximum growth at 25℃. Mycelial growth of *A. subrufescens* (KH 407) was stimulated at lower temperature of 20℃. Results are in close correspondence to Hernández et al. (2011) who determined 20–25℃ temperature range as optimum for mycelial growth of wild *Agaricus subrufescens* strains. Strains varied in efficiency and spawn running depending upon the quality of mycelial growth. Rashid et al. (2018) utilized compost with Egyptian pea straw, wheat straw, $CaSO_4$, horse manure and phosphate for *A. bisporus* to check mycelial growth rates. Llarena et al. (2014) investigated use of wheat straw based commercial compost for spawn running of *A. subrufescens* in Brazil. Similarly, in this study wild strains of *A. subrufescens* (KH 407) were tested for spawn running on mushroom compost. Commercial strain of *Agaricus bisporus* has been

cultivated in Mexico by Salmones et al. (2018). The pin head emergence was observed after 36 days of spawning, at 25℃, followed by period of 6-8 days required for the maturation of basidiocarp. Contrarily, in this study pin heads of *A. bisporus* W-01 appeared after 7–10 days of casing application at 20℃ and maximum development of basidiocarp was obtained in 5-7 days. Difference in time period might be due to difference in the incubation temperatures, as well as strain used in this study was wild. Primordial formation was observed for *A. subrfescens* (KH 407) strain in 2 months after peat moss casing. Our results are in contradiction to Zied et al. (2012) who reported that, primordial formation of *A. subrufescens* was observed in 22 days after application of loamy soil casing. Conditions for cultivation of *A. subrufescens* in Brazil described by Zied et al. (2018) suggested the optimum humidity levels between 85–90%. Delayed pin head formation in our strain might be due slower mycelial growth rates as observed for this strain as well as humidity levels were not maintained up to 80%. Moreover, casing of peat moss was applied in contrast to loamy soil. The present study shows that compost extract agar medium is suitable for the culturing of indigenous *Agaricus bisporus* and *A. subrufescens*. For spawn production, sorghum grains proved as better alternative to wheat. Low and high temperature strains of both *Agaricus* species were analyzed during this research work. *Agaricus bisporus* and *A. subrufescens* were cultivated using soil and peat moss as casing material, respectively. Higher yield was observed for *Agaricus bisporus*. Hence, it is concluded that the wild strains of indigenous *Agaricus* species have the ability to yield good mycelial growth for culture bank and spawn production. These strains are beneficial as they can adapt to local environmental conditions with slight modifications in temperature and moisture.

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