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RESEARCH ARTICLE

STUDIES ON THE INFLUENCE OF CARBON AND NITROGEN NUTRITION ON THE CHLAMYDOSPORES PRODUCTION OF *VOLVARIELLA VOLVACEAE* (BULL. EX FR.) SING. AND *VOLVARIELLA BOMBYCINA* (SCHAEFF.) SINGER

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ABSTRACT

Carbon sources *viz.,* glucose, fructose, sucrose, mannitol and sorbitol; Nitrogen sources *viz.,* ammonium nitrate, sodium nitrate, potassium nitrate, glycine and calcium nitrate were supplemented in Czapek's dox medium to study their influence on chlamydospores production of Volvariella. Supplementation of sucrose and potassium nitrate to the growth medium each at 3 per cent level encouraged the mycelial growth and chlamydospores production of both *V. volvacea* strain CBE TNAU 1505 and *V. bombycina* strain CBE TNAU 1406.

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INTRODUCTION

Mushrooms are myriad of nutritious healthy foods that are valued throughout the world as luscious medicine for thousands of years. Among the edible mushrooms, *Volvariella volvacea* (Bull. Ex Fr.) Sing. is known as paddy straw mushroom or Chinese mushroom that grows in tropical and sub-tropical regions at a temperature range of 28-35^{0}C. *V. bombycina* (Schaeff.) Singer is popularized as silver silk straw mushroom or tree mushroom grows in temperate conditions at a temperature range of 23-25^{0}C (Ahlawat and Singh, 2014). Poor shelf life, hydrolytic enzyme potential and productivity are the major hindrances for this praise worthy mushroom for not gaining commercial importance in India. Genome sequencing of Volvariella suggests that it is a secondarily homothallic fungus that produces brown, thick walled and multinucleated asexual chlamydospores and sexual basidiospores (Chang, 1969 and Bao *et al.,* 2013). Chlamydospores are produced by many mushroom fungi and represent thick walled vegetative cells with varied forms of condensed cytoplasm normally formed at hyphal tips (on apical cells) or within hyphae. Commonly, chlamydospores of Volvariella are uniformly thickened spherical with an average diameter of 58.8 µ (Chang and Yau, 1971). Monosporous isolates of paddy straw mushroom vary in their growth rate, abundance of mycelia, aerial hyphae and presence of chlamydospores. Chang *et al.,* 1981. The selection of strains is normally done based on the earliness in mycelial growth and chlamydospores production. A positive correlation has been established with the yield potential and chlamydospores production of *Volvariella* (Chang, 1972). Mannitol contributes up to 20 per cent of the mycelial dry weight which further increases dramatically to 30-50 per cent in differentiating sporophores (Stoop and Mooibroek, 1998). Complex organic nitrogen sources like yeast extract, peptone, tryptophan, aspartic acid, serine and casein hydrolysate; inorganic nitrogen sources such as ammonium di-hydrogen phosphate were found to be the best for protein production, germination and germ tube elongation of the spores of *V. diplasia* (Banerjee and Samajpati, 1989; Banerjee *et al.,* 1990). Keeping this bounded facts in hark, the present investigation has been made for selecting the best nutritional parameters for enhancing chlamydospores production of Volvariella.

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MATERIALS AND METHODS

V. volvacea strain CBE TNAU 1505 and V. *bombycina* strain CBE TNAU 1406 were obtained from the germplasm bank of Mushroom Research Laboratory, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India was used in this experiment. It was maintained on 90 mm petridishes with potato dextrose agar medium at 32± 2^0C.

Maintenance of Pure Cultures

The sub-cultures of paddy straw mushroom strains used in the study were maintained on Potato Dextrose Agar (PDA) medium. In order to maintain the vigour fresh isolations were made from the fruiting bodies every time after 2 to 3 subcultures. For this purpose the strains were propagated in straw spawn and grown on paddy straw following the method suggested by Thomas *et al.* (1943). Freshly harvested sporophores were swabbed with 70 per cent ethanol. At the junction of the pileus and stipe, tissue bits were removed aseptically, surface sterilized with 70 per cent ethanol for 30 sec and repeatedly washed in sterile water and placed on PDA medium taken in sterile Petri dishes. The dishes were incubated at 30 to 35ºC for 7 d. Following single hyphal tip method (Rangasamy, 1972) pure cultures were made and stored in PDA slants to carry out further studies.

Effect of Carbon (C) and Nitrogen (N) Nutrition

To select the best source of carbon and nitrogen nutrition that would induce the growth and chlamydospores formation of *V. volvacea* strain CBE TNAU 1505 and *V. bombycina* strain CBE TNAU 1406, some of the C sources like glucose, fructose, sucrose, mannitol and sorbitol and N sources like ammonium nitrate, sodium nitrate, potassium nitrate, glycine and calcium nitrate were substituted separately in the basal Czapek's dox medium (Thom and Raper, 1945). Conical flasks containing the nutrient substituted medium were steam sterilized at 1.46 kg / cm^2 for 20 min. After cooling, 0.3 ml of streptomycin sulphate (500 ppm) was incorporated to 100 ml of the medium to avoid bacterial contamination. The medium was aseptically poured in 90 mm Petri dishes. A 9 mm mycelial disc of specific strain was separately inoculated aseptically in replicates on medium amended with respective carbon and nitrogen sources. The inoculated Petri dishes were incubated at 30 - 35°C. Visual observations on the radial mycelial growth, colony characters, and formation of chlamydospores were recorded.

Statistical Analysis

Statistical software AGRES (Developed by Dept. of Physical Science, TNAU, Coimbatore) was used for analysis of data obtained in the experiment. All the visual observation parameters were carried out in 4 replications whereas; micrometric observation parameters were carried out in 25 replications. In case of zero values the data was log transformed (X+0.5) before statistical analysis.

RESULTS AND DISCUSSION

Five carbon and nitrogen sources were used to check the growth and chlamydospores production of *V. volvacea* strain CBE TNAU 1505 and *V. bombycina* strain CBE TNAU 1406. The readings were taken at sixth day after inoculation in case of CBE TNAU 1505 and at eighth day after inoculation in case of CBE TNAU 1406.

Carbon nutrition and chlamydospores production

Various kinds of monosaccharides, disaccharides and sugar alcohols were used as carbon sources to test their influence on chlamydospores production of *V. volvacea* and *V. bombycina* strain CBE TNAU 1505 and CBE TNAU 1406. Among which, sucrose amended medium had shown the maximum radial growth of 88.2 mm and complete growth of 90 mm was reached in 7.5 d, followed by sorbitol (81.7 mm and 8.9 d) and mannitol (42.1 mm and 12.6 d) amendments. Fructose supplemented medium showed minimum radial growth of 28.3 mm, which had taken 22.6 d for completing 90 mm growth in Petri plates. Sucrose amended medium took 14.5 d for chlamydospores formation followed by sorbitol (15.2 d), mannitol (15.8 d), dextrose (19.9 d) and fructose (24.2 d), respectively. In case of *V. bombycina* strain CBE TNAU 1406, sucrose amended medium showed the maximum radial growth of 89.5 mm and reached the growth of 90 mm in Petri plates in 8.6 d, followed by sorbitol (88 mm and 9.1 d) and mannitol (77 mm and 9.7 d). Dextrose amended medium showed minimum radial growth of 46.7 mm, which took 12.9 d for completing 90 mm growth. Sucrose amended medium took 7.8 d for chlamydospores formation followed by sorbitol (8.3 d), mannitol (8.7 d), fructose (10.2 d) and dextrose (11.4 d), respectively. The results of the experiment are presented in Table 1 and 2 and the microscopic observations were presented in Fig 1 and 2. In the present inquest sucrose was found to be best carbon source for chlamydospores production and growth of both the strains, followed by sorbitol. The results partially support the findings of Rangasamy (1956), who had tested various carbon sources to induce the growth and morphogenesis of *V. diplasia* and revealed that the highest biomass production was from starch supplemented broth followed by sucrose. Besides, Prabhu (2006) found that sugar alcohols such as sorbitol and mannitol greatly encouraged the biomass and chlamydospores production by *V. volvacea* strain PS1. Jyothi and Anitha (2014) accustomed sucrose rich substrates such as sugar cane baggase along with paddy straw (1:1) to increase the yield of *Volvariella* sp. This work further substantiates the superiority of sucrose as C source for the growth and chlamydospores production of *Volvariella* spp.

Nitrogen nutrition and chlamydospores production

Among the different N sources evaluated with *V. volvacea* strain CBE TNAU 1505, potassium nitrate (89.5 mm, 6.6 d), ammonium nitrate (86.2 mm, 7.3 d) and calcium nitrate (83.7 mm, 8.4 d) were found to be on par in terms of radial growth and days taken to cover 90 mm in Petri plates, respectively, followed by sodium nitrate (73.7 mm, 9.8 d); whereas, minimum growth was observed in glycine amended medium (57.2 mm, 11.4 d). KNO_3 amended medium showed the chlamydospores formation in 13.6 d, followed by NH_4NO_3 (14.3 d) and $CaNO_3$ (15.2 d). Glycine amended medium had shown chlamydospores after 17.2 DAI. In case of *V. bombycina* strain CBE TNAU 1406, KNO_3 (90mm, 9.4 d), NH_4NO_3 (89.5 mm, 10.5 d) and $CaNO_3$ (89.2 mm, 11.2 d) were found to be on par in terms of radial growth and days taken to complete 90 mm in Petri plates.

Table 1. Carbon nutrition and chlamydospores production by *V. volvacea* strain (CBE TNAU 1505)

C source	Radial growth in mm* (6 DAI)	DTTCPP*	Aerial hyphae*	Colony morphology*	DTFCP*	Chlamydospores density*	Micrometric observations#	
							Hyphal diameter (µm)	Chlamydospore diameter (µm)
Mannitol	42.1[c]	12.6[c]	++	Thin transparent radiating	15.8[b]	++	6.0	26.4
Sorbitol	81.7[b]	8.9[b]	+++	Thick projecting	15.2[b]	++	5.2	24.0
Dextrose	35.0[d]	14.5[d]	+	Thin strandy	19.9[c]	+	4.7	22.5
Fructose	28.3[e]	22.6[e]	-	Thin transparent	24.8[d]	+	4.3	23.8
Sucrose	88.2[a]	7.5[a]	++++	Thick fluffy	14.5[a]	+++	5.8	25.3
CD (P = 0.05)	4.8	0.4			0.6			

Aerial hyphae, chlamydospores density "- to ++++" absent to highly densed, DTTCPP - Days taken to cover 90 mm Petri plate. DTFCP – Days taken for chlamydospores production. *, # Values are mean of 4 and 25 replications. Means followed by a common letter are not significantly different at P = 0.05 by one way ANOVA.

Table 2. Carbon nutrition and chlamydospores production by *V. bombycina* strain (CBE TNAU 1406)

C source	Radial growth in mm* (8 DAI)	DTTCPP*	Aerial hyphae*	Colony morphology*	DTFCP*	Chlamydospores density*	Micrometric observations#	
							Hyphal diameter (µm)	Chlamydospore diameter (µm)
Mannitol	77.0[b]	9.7[c]	+	Thin transparent irregularly projecting	8.7[c]	+++	5.7	24.2
Sorbitol	88.0[a]	9.1[b]	+	Thin uniformly projecting	8.3[b]	+++	5.0	24.0
Dextrose	46.7[c]	12.9[e]	-	Thin wavy	11.4[e]	++	4.6	26.4
Fructose	71.7[b]	12.4[d]	-	Thin	10.2[d]	++	4.9	25.1
Sucrose	89.5[a]	8.6[a]	++	Thin fluffy	7.8[a]	++++	5.9	26.7
CD (P = 0.05)	8.1	0.3			0.3			

Aerial hyphae, chlamydospores density "- to ++++" absent to highly densed. DTTCPP – Days taken to cover 90 mm Petri plate. DTFCP – Days taken for chlamydospores production. *, #Values are mean of 4 and 25 replications. Means followed by a common letter are not significantly different at P = 0.05 by one way ANOVA.

Table 3. Nitrogen nutrition and chlamydospores production by *V. volvacea* strain (CBE TNAU 1505)

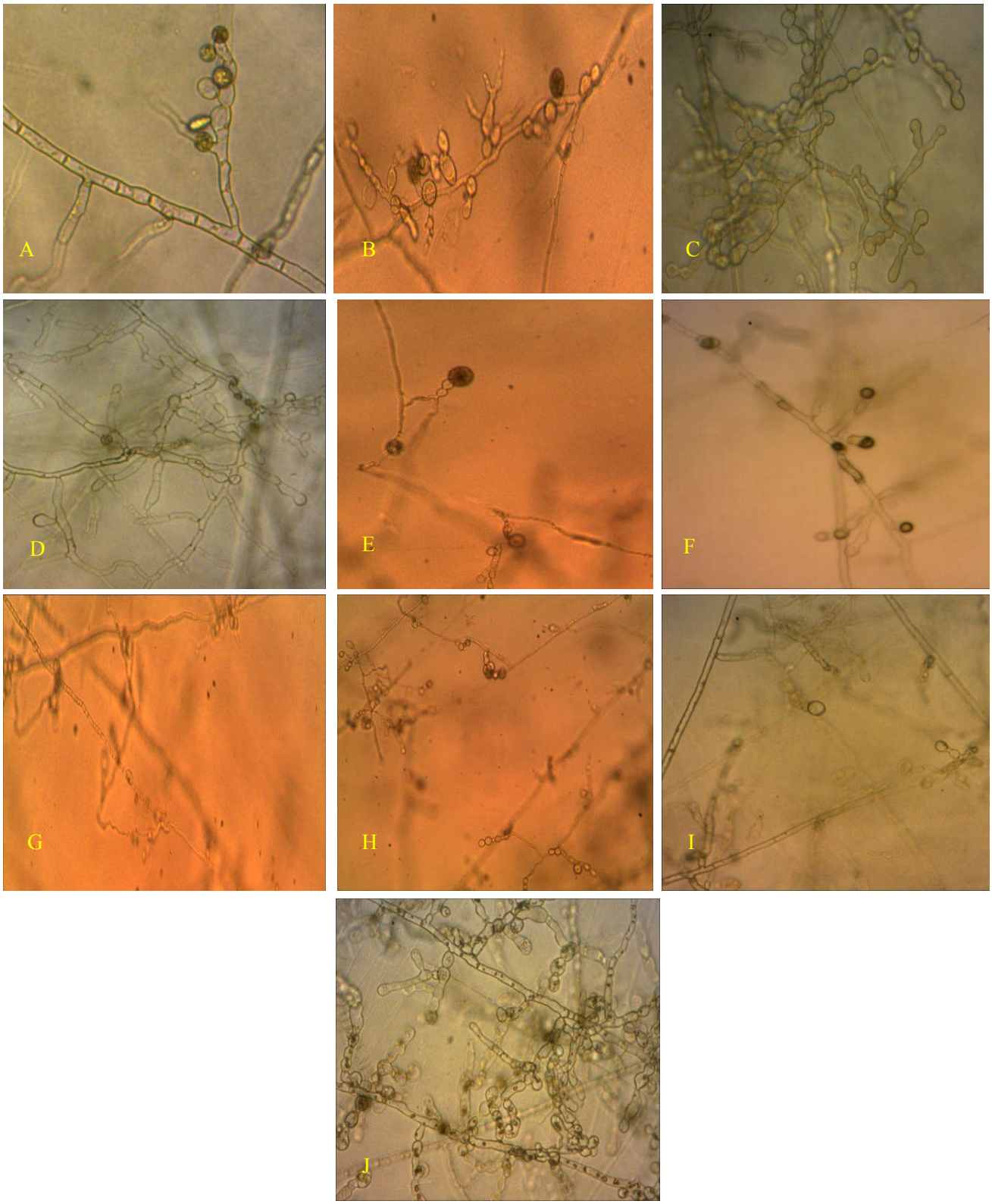
N source	Radial growth in mm* (6 DAI)	DTTCPP*	Aerial hyphae*	Colony morphology*	DTFCP*	Chlamydospores density*	Micrometric observations#	
							Hyphal diameter (µm)	Chlamydospore diameter (µm)
Ammonium nitrate	86.2[a]	7.3[b]	++	Thick strandy	14.3[b]	+++	5.6	25.4
Calcium nitrate	83.7[a]	8.4[c]	-	Thin transparent	15.2[c]	++	5.1	25.8
Potassium nitrate	89.5[a]	6.6[a]	++++	Thick fluffy	13.6[a]	++++	6.0	26.2
Sodium nitrate	73.7[b]	9.8[d]	-	Thin radiating	15.9[d]	++	4.9	21.2
Glycine	57.2[c]	11.4[e]	+	Highly thin transparent	17.2[e]	+	4.7	23.8
CD (P = 0.05)	8.2	0.3			0.6			

Aerial hyphae, chlamydospores density "- to ++++" absent to highly densed. DTTCPP – Days taken to cover 90 mm Petri plate. DTFCP – Days taken for chlamydospores production. *, #Values are mean of 4 and 25 replications. Means followed by a common letter are not significantly different at P = 0.05 by one way ANOVA.

Table 4. Nitrogen nutrition and chlamydospores production by *V. bombycina* strain (CBE TNAU 1406)

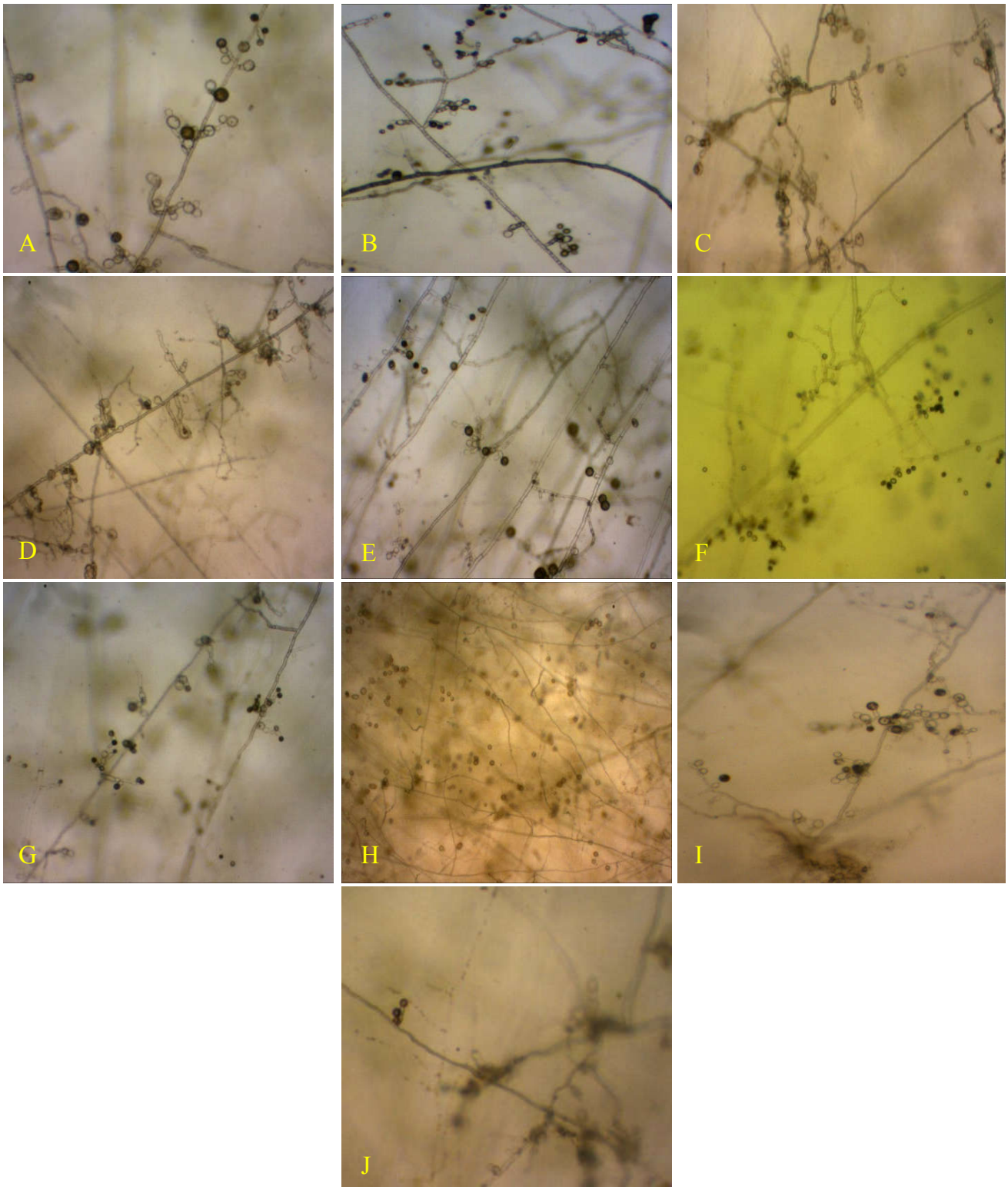
Nitrogen source	Radial growth in mm* (8 DAI)	DTTCPP*	Aerial hyphae*	Colony morphology*	DTFCP*	Chlamydospores density*	Micrometric observations#	
							Hyphal diameter (µm)	Chlamydospore diameter (µm)
Ammonium nitrate	89.5[a]	10.5[b]	+++	Thick	9.2[b]	+++	5.7	26.1
Calcium nitrate	89.2[a]	11.2[c]	+	Thin transparent	9.9[c]	++	5.4	24.5
Potassium nitrate	90.0[a]	9.4[a]	++	Thick strandy	8.2[a]	++++	6.6	24.8
Sodium nitrate	79.5[b]	12.7[d]	-	Thin transparent	11.7[d]	++	6.3	25.2
Glycine	75.5[c]	13.5[e]	-	Thin irregular transparent	13.7[e]	+	4.8	23.4
CD (P = 0.05)	4.2	0.4			0.3			

Aerial hyphae, chlamydospores intensity "- to ++++" absent to highly densed. DTTCPP – Days taken to cover 90 mm Petri plate. DTFCP – Days taken for chlamydospores production.
*, #Values are mean of 4 and 25 replications. Means followed by a common letter are not significantly different at P = 0.05 by one way ANOVA.



A - Mannitol
B - Sorbitol
C - Dextrose
D - Fructose
E - Sucrose
F - Ammonium nitrate
G - Sodium nitrate,
H - Potassium nitrate
I - Calcium nitrate
J - Glycine.

Fig 1. Carbon and Nitrogen nutrition and chlamydospore production of *V. volvacea* strain CBE TNAU 1505



A - Mannitol
B - Sorbitol
C - Dextrose
D - Fructose
E - Sucrose
F - Ammonium nitrate
G - Sodium nitrate
H - Potassium nitrate
I - Calcium nitrate
J - Glycine.

Fig 2. C and N nutrition and chlamydospore production of *V. bombycina* strain CBE TNAU 1406

This was followed by $NaNO_3$ (79.5 mm, 13.5 d). The least mycelial growth was observed in the medium amended with glycine (75.5, 12.7 d). KNO_3 amended medium showed chlamydospores formation in 8.22 d followed by NH_4NO_3 (9.2 d) and $CaNO_3$ (9.9 d). Glycine amended medium showed chlamydospores after 13.7 DAI. The results of the experiment are presented in Table 3 and 4 and the microscopic observations were presented in Fig 1 and 2. Nitrogen is the prime constituent of the amino acids which make up the proteins and polysaccharide chitin, a cell wall component of many fungi (Chang and Quimio, 1982). Darlington and Scazzocchio (1967) chronicled that if one nitrogen source was utilized by a fungus for a long time, the pH of the medium would rise. Later, Sprent (1987) first reported the association between pH and nitrogen metabolism. Amidst the different nitrogen sources used the strains CBE TNAU 1505 and CBE TNAU 1406 preferred potassium nitrate followed by ammonium nitrate for mycelial growth and chlamydospores formation. But, glycine was found to be the best nitrogen source for biomass production and chlamydospores density by Prabhu (2006). Kalra *et al*. (1997) perspicuously indicated that organic nitrogen sources like asparagine and glutamic acid were the best sources for the growth of *V. diplasia*. Normally, some fungus prefers nitrate form of nitrogen while, others be partial to ammonical form still, others can utilize neither nitrate nor ammonia and may require an organic nitrogen containing compound. This requisite for organic nitrogen may actually be a requirement for a specific aminoacid. A fungus that can utilize nitrate will also harness ammonium and organic nitrogen compounds for its nitrogen requirements (Chang and Miles, 1982). Fascinatingly, in the present investigation, *V. volvacea* strain CBE TNAU 1505 and *V. bombycina* strain CBE TNAU 1406 flaunted preference for both nitrate and ammonical form of nitrogen (potassium nitrate followed by ammonium nitrate). When nitrate nitrogen in the substrate was preferred by the organism, it resulted in alkaline pH of the medium by the release of excess cations. In contrast, preference of ammonical nitrogen resulted in acidic medium by the increased release of anions (Kurtzman and Chang, 1982). Hereby, further study by involving different carbon and nitrogen sources on the chlamydospores production is much warranted for commercial exploitation of Volvariella.

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