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Effect of Physical Factors, Carbon and Nitrogen Sources on the Growth and Sclerotial Production in *Morchella Hybrida* (Sow.) Pers.

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ABSTRACT: The *in vitro* studies on physical and nutritional requirements (Carbon and Nitrogen sources) for the growth and sclerotial production in *Morchella hybrida* (Sow.) Pers. have revealed that this morel grows and reproduces best at an optimum temperature of 24˚C and pH 7.0 after ten days of incubation on Czapek's medium. The growth and sclerotia production on nine different carbon compounds *viz.* D(+)xylose, D(+)glucose, D(-)fructose, L(-)sorbose, Maltose, Lactose, Sucrose, Raffinose and Starch was investigated. Out of these nine carbon sources, D(+)xylose have been found to be the best for growth and sclerotia production. Twenty six different nitrogen compounds *viz.* Potassium nitrate, Sodium nitrate, Sodium nitrite, Ammonium acetate, Ammonium oxalate, Ammonium chloride, Ammonium sulphate, Ammonium phosphate, Ammonium nitrate, L-arganine, L-cysteine HCl, DL-aspartic acid, L-cysteine, L-glutamic acid, Glycine, L-histidine HCl, L-leucine, DL-methionine, L-ornithine HCl, L-β-phenylalanine, L-proline, DL-serine, L-tyrosine, DL-valine, DL-tryptophan and DL-threonine have been used for the nitrogen nutrition studies. Out of these twenty six nitrogen sources, the fungus produces maximum mycelial growth with L-glutamic acid and the best production of sclerotia with L-glutamic acid and L-ornithine HCl.

Keywords: Sclerotia; *Morchella hybrida*; Czapek's medium and Morels.

INTRODUCTION: Morels are wild edible ascomycetous mushrooms belonging to the family Helvellaceae. Fructifications (Ascocarps) of bodies of morel fungi (*Morchella* spp.) are highly valued for their medicinal and nutritional qualities.[1] Ower was the first to produce *Morchella esculenta* ascocarps *in vitro*.[2] Investigations have shown that there is a stage in the life cycle of morels called the sclerotium. It is also experimentally demonstrated that sclerotia are essential in production of fructifications under controlled conditions.[3] Various researchers have contributed greatly for the *in vitro* production of sclerotia that can be employed for the production of ascocarps.[4,5,6,7,8,9,10,11,12,13,14&15] Inspite of all these efforts not much has been achieved regarding the commercial cultivation of *M. esculenta*. *Morchella hybrida* is another excellent species which is popular among the local people in Himachal Pradesh state of India for its edibility and aroma. It is envisaged to devise technology for the cultivation of this species. Since the knowledge of nutritional requirements is a pre-requisite to study the physiological parameters of ascocarp development, studies were initiated to determine the nutritional parameters for the growth and reproduction of *M. hybrida.*

MATERIAL AND METHODS: The ascocarp of *M. hybrida* were collected from the forests of Himachal Pradesh, India. The identification of the species was made after studying the fresh and dried specimens and comparing it with the standard descriptions.[16] The dried specimens were revived in 2% KOH. The microscopic details were studied in cotton blue (lactic acid - 30g, cotton blue - 0.05g). The single hyphal tip cultures were isolated from the ascocarps selected at random and maintained on solidified MEA (malt extract agar medium) at 0-4˚C, to be employed for further studies. All the experiments were performed in liquid basal medium found optimum for growth and reproduction. The basal media was autoclaved at 15lb psi steam pressure for 15 minutes. The pH of the medium was adjusted with sterilized solution of 0.1N KOH/0.1N HCl after sterilization.[17] The pH adjustments were checked over P/L Philips precision instrument PR 9045 M. The 25 ml of the basal media were apportioned in each sterilized 100 ml conical

Erlenmeyer flask. Three replicates were kept for each variable in an experiment. The inoculums consisted of discs cut from a rapidly growing margin of petriplate culture (10 days old) grown on the MEA, with the help of cork-borer and used to inoculate each flask. The following observations of the individual replicate were recorded at the end of each experiment.

Mycelial dry weight and final pH of the medium: At the end of each experiment the mycelium of individual replicate was filtered through previously weighed goosch crucible under suction, dried at 60˚C in hot air oven to a constant weight. The final pH was checked over P/L Philips precision instrument PR 9045 M.

Sclerotial production: The presence and absence of sclerotia in the cultures of all variables were noted visually and have been designated as – (for nil), + (for fair), ++ (for good) and +++ (for the best) production of sclerotia.

Statistical analysis of data: The data was analyzed statistically by randomized block system from dry weight of individual replicates with variables in terms of significance and insignificance of data. The significance is appended to each table and denoted by statistical error (SE), statistical error of difference (SE_d) and critical difference (CD).

RESULTS AND DISCUSSION:

Growth and sclerotial production with different basal media: In this experiment an account of growth (average mycelia dry weight -mg/25ml) and sclerotial production was studied with 12 different basal media. The initial pH of the different basal media was checked before and after autoclaving. The fungus was incubated at 24˚C for ten days.

Table 1: Growth and sclerotial production of *Morchella hybrida* with different basal media at 24˚C after ten days of incubation.

Liquid Basal Media	Initial pH	Final pH	Average mycelial dry weight (mg/25ml)	Sclerotial production
Richard's	4.25	4.91	95.3	-
Raulin's	2.42	3.52	60.6	-
Dox's	4.73	5.51	64.3	-
Czapek's-I	4.88	5.22	109.0	+
Czapek's-II	4.75	5.10	136.3	+
Coon's	5.32	5.33	26.6	-
Glucose peptone	4.77	5.35	85.3	+
Elliot's	6.29	7.21	114.6	-
Glucose nitrate	4.42	5.40	63.6	+
Brown's-I	5.32	5.40	62.0	-
Brown's-II	5.35	4.58	70.0	-
Asthana & Hawker's	4.87	5.22	76.3	+
Replicates: Insignificant, Variables: Significant, SE: 1.2, SE_d: 1.7, CD (5%): 3.4				

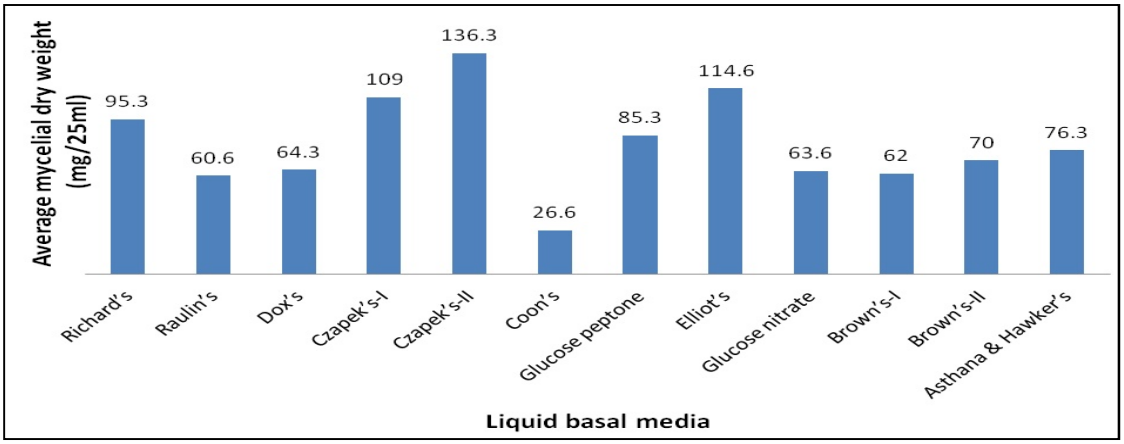

Figure 1: Growth and sclerotial production of *Morchella hybrida* with different basal media at 24°C after ten days of incubation.

The data on growth, sclerotial production and mean final pH of the cultural filtrate was recorded. The statistical analysis of the data on growth of each replicate and the variable is appended to Table 1 and Figure 1. The optimum mycelia growth occurred on Czapek's-II medium, whereas sclerotial production occurred with Czapek's-I & II, Glucose-peptone, Glucose nitrate and Asthana & Hawker's medium. The fungus formed light brown to dark brown, submerged to superficial and incomplete to complete mycelia mat with different basal media. The final pH of the different basal media did not changed significantly with the growth of the fungus.

Relationship with temperature: The fungus was grown on Czapek's-II medium and studied with selected temperatures of 20, 24, 28 and 32˚C. The inoculum disc was cut from the growing margin of a ten days old colony and inoculated in each flask. Three replicates were kept for each temperature under aseptic conditions. The data on growth, sclerotial production and mean final pH of the cultural filtrate was recorded. The statistical analysis of the data on growth of each replicate and the variable is appended to Table 2 and Figure 2. The optimum mycelial growth and good amount of sclerotial production occurred at 24˚C. Fair amount of sclerotial production occurred at 20˚C whereas no sclerotial formation occurred at 28˚C and 32˚C. The final pH shifted significantly with all these temperatures. The fungus formed incomplete, submerged to superficial and light brown mycelial mat at 20˚C, 28˚C and 32˚C, whereas it formed complete, submerged to superficial and dark brown mycelial mat at 24˚C.

Table 2: Growth and sclerotial production of *Morchella hybrida* at different temperature (20-32˚C) after ten days of incubation

Temperature (˚C)	Final pH	Average mycelial dry wt. (mg/25ml)	Sclerotial production
20	5.70	122.2	+
24	5.50	147.0	++
28	4.99	92.3	-
32	4.58	62.0	-
Replicates: Insignificant, Variables: Significant, SE: 1.4, SE_d: 1.98(2), CD (5%): 4.57			

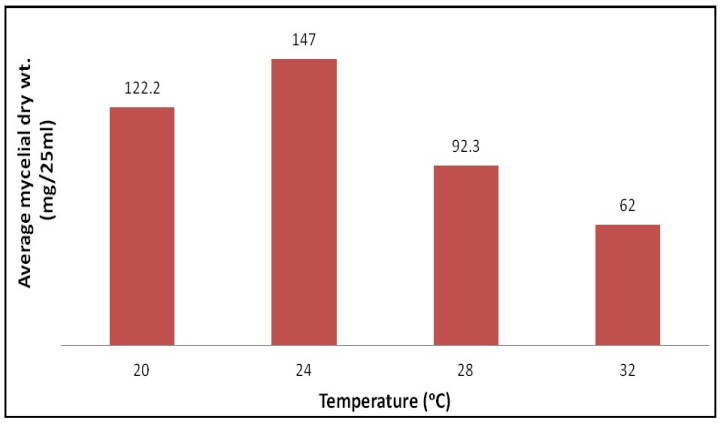

Figure 2: Growth and sclerotial production of *Morchella hybrida* at different temperature (20-32°C) after ten days of incubation.

Relationship with H-ion concentrations: The fungus was studied with selected H-ion concentrations (3, 4, 5, 6, 7 and 8) on Czapek's medium at 24˚C for ten days. Three replicates were kept. The data on growth, sclerotial production and mean final pH of the cultural filtrate was recorded. The statistical analysis of the data on growth of each replicate and the variable is appended to Table 3 and Figure 3. The best mycelia growth and sclerotial production occurred at pH 7, whereas good amount of sclerotial production occurred at pH 6 and pH 8. Fair amount of sclerotial production occurred at pH 5, whereas no sclerotial formation occurred at pH 3 and pH 4. The fungus formed light brown to brown, submerged and incomplete mycelial mat with pH 3, 4 and 5. It formed complete, submerged to superficial and light brown to dark brown mycelial mat with pH 6, 7 and 8.

Relationship with days of incubation: A period of 16 days was selected to observe the optimum days of incubation for the growth and sclerotial production. The basal pH was adjusted to an optimum of pH 7 and mycelial disc was inoculated in each flask at temperature 24˚C. Three replicates were kept for each treatment. The data on mycelial growth, sclerotial production and mean final pH of the cultural filtrate was recorded. The statistical analysis of the data on growth of each replicate and the variable is appended to Table 4 and Figure 4. The fungus attained maximum growth after ten days of incubation, thereafter the growth declined. Fair production of sclerotia occurred upto 8th day, best amount of sclerotia were produced upto 12th day and good amount of sclerotia were produced from 14th-16th days of incubation. The fungus formed submerged, incomplete, light brown mycelial mat upto 8th day and thereafter it formed complete, submerged to superficial and dark brown mycelial mat from 9th-16th day of incubation.

Table 3: Growth and sclerotial production of *Morchella hybrida* at different H-ion concentrations (pH 3 to pH 8) at temperature 24˚C after ten days of incubation.

Initial pH	Final pH	Average mycelia dry wt. (mg/25ml)	Sclerotial production
3	2.92	23.0	-
4	3.48	76.3	-
5	4.22	118.6	+
6	5.89	136.7	++
7	7.33	149.3	+++
8	7.28	114.0	++
Replicates: Insignificant, Variables: Significant, SE: 1.4, SEd: 1.9, CD (5%): 4.4			

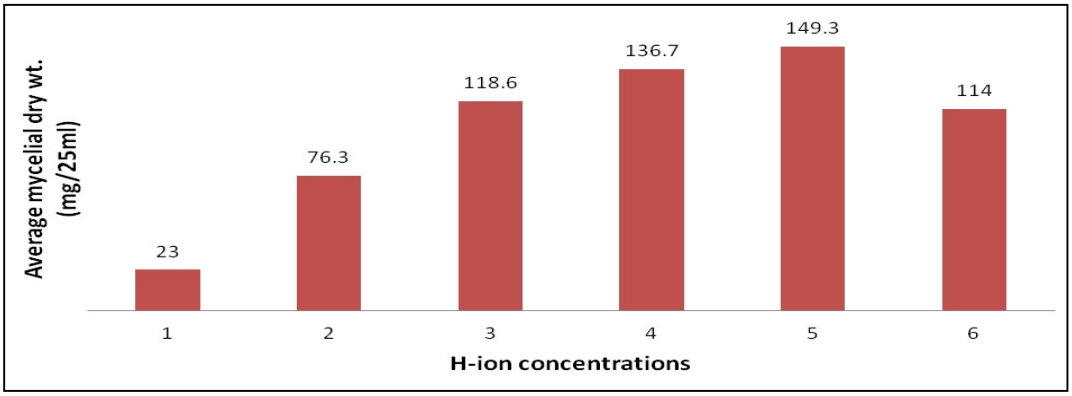

Figure 3: Growth and sclerotial production of *Morchella hybrida* at different H-ion concentration (pH 3-pH 8) at temperature 24°C after ten days of incubation.

Table 4: Growth and sclerotial production of *Morchella hybrida* at different days of incubation at temperature 24˚C and pH 7.0.

Days of incubation	Final pH	Average mycelial dry wt. (mg/25ml)	Sclerotial production
2	6.99	32.0	-
4	6.98	54.6	-
6	6.98	88.3	-
8	7.03	118.6	+
10	7.04	148.0	+++
12	7.11	127.0	+++
14	7.05	116.0	+++
16	7.03	98.3	++
Replicates: Insignificant, Variables: Significant, SE: 1.66, SE_d: 2.35, CD (5%): 4.82			

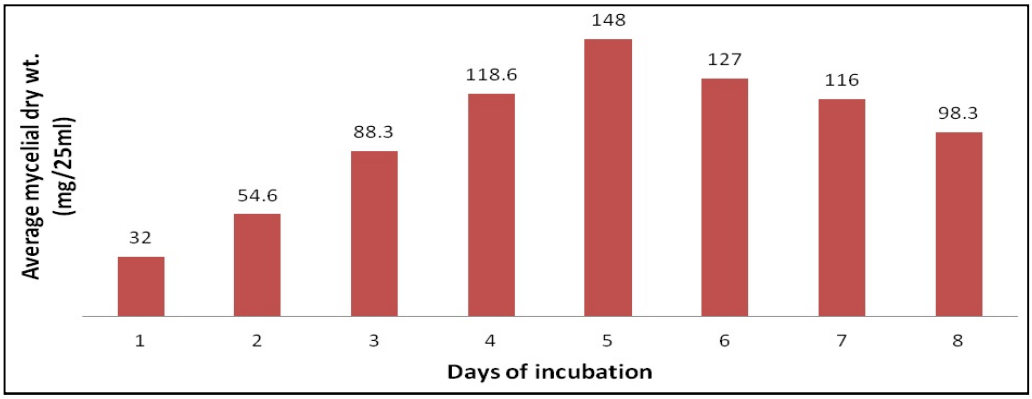

Figure 4: Growth and sclerotial production of *Morchella hybrida* at different days of incubation at temperature 24°C and pH 7.0.

Relationships with different Carbon sources: Nine carbon compounds (comprising 4 monosaccharide, 4 oligosaccharide, 1 polysaccharide) were tested singly on selected basal medium at temperature 24˚C, pH 7 and for ten days of incubation. Each carbon compound served as the sole source of carbon for the growth and sclerotial production by the fungus. Sucrose of the basal medium was substituted singly by each of these carbon compounds so as to provide 12 g/l of carbon, which is the amount present in 28.5 g of sucrose. Three replicates were kept for each carbon compound. The data on mycelial growth, sclerotial production and mean final pH of the cultural filtrate has been recorded after ten days of incubation. The statistical analysis of the data on growth is appended to Table 5 and Figure 5. The fungus produced optimum mycelial growth with D(+) xylose followed by D(+)glucose > sucrose > D(-)fructose > D(-)raffinose > maltose > starch > lactose > D(-)sorbose. The fungus gave best production of sclerotia with D(+) xylose, whereas it gave good production of sclerotia with D(+)glucose, sucrose and starch. There was nil production of sclerotia occurred with lactose and D(-)sorbose.

Table 5: Growth and sclerotial production of *Morchella hybrida* with different carbon compounds at temperature 24˚C, pH 7.0 and after ten days of incubation.

Carbon compounds	Final pH	Average mycelial dry wt. (mg/25ml)	Sclerotial production
Control	7.12	30.0	-
D(+)xylose	8.21	220.2	+++
D(+)glucose	7.36	199.5	++
D(-)fructose	7.58	165.0	+
L(-)sorbose	5.50	60.2	-
Maltose	7.10	152.3	+
Lactose	7.53	105.0	-
Sucrose	8.04	178.2	++
Raffinose	7.22	159.1	+
Starch	7.40	150.5	++
Replicates: Insignificant, Variables: Significant, SE: 1.8, SE_d: 2.6, CD(5%): 5.4			

Inorganic nitrogenous compounds: Nine inorganic nitrogen compounds were tested for the growth and sclerotial production. Sodium nitrate of the basal medium was substituted singly with each inorganic nitrogen compounds so as to provide 0.250 g of nitrogen, an amount present in 1.5 g of $NaNo_3$. Three replicates were kept for each nitrogen compound. The data on mycelial growth, sclerotial production and mean final pH of the cultural filtrate was recorded after ten days of incubation. The statistical analysis of the data on growth is appended Table 6 and Figure 6. The fungus produced maximum mycelial growth with ammonium sulphate followed by ammonim nitrate > sodium nitrite > ammonium phosphate > sodium nitrate > potassium nitrate > ammonium chloride > ammonium acetate > ammonium oxalate. The fungus gave best production of sclerotia with ammonium sulphate and ammonium nitrate whereas good production of sclerotia with ammonium phosphate. There was nil production of sclerotia with potassium nitrate, ammonium acetate and ammonium oxalate.

Table 6: Growth and sclerotial production of *Morchella hybrida* with different inorganic nitrogenous compounds at temperature 24˚C, pH 7.0 and after ten days of incubation.

Inorganic nitrogen compounds	Final pH	Average mycelial dry wt. (mg/25ml)	Sclerotial production
Control	6.90	30.2	-
Potassium nitrate	7.02	140.3	+
Sodium nitrate	6.89	150.0	+
Sodium nitrite	6.88	165.0	+
Ammonium acetate	5.22	80.2	-
Ammonium oxalate	6.85	70.1	-
Ammonium chloride	6.12	132.0	+
Ammonium sulphate	6.55	192.0	+++
Ammonium phosphate	6.88	159.0	++
Ammonium nitrate	7.03	177.0	+++
Replicates: Insignificant, Variables: Significant, SE: 1.39, SE_d: 1.97, CD(5%): 4.0930			

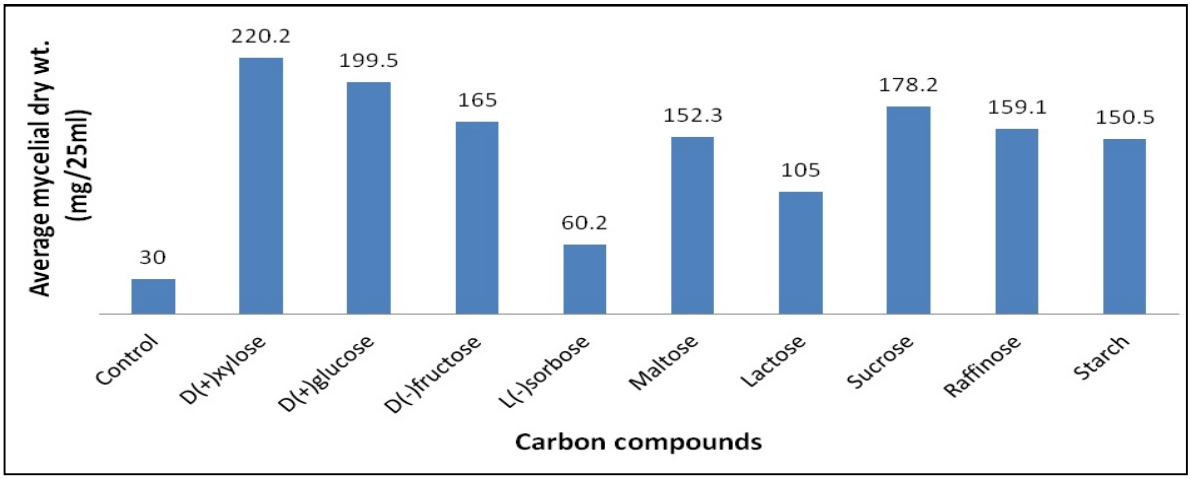

Figure 5: Growth and sclerotial production of *Morchella hybrida* with different carbon compounds at temperature 24°C, pH 7.0 and after ten days of incubation.

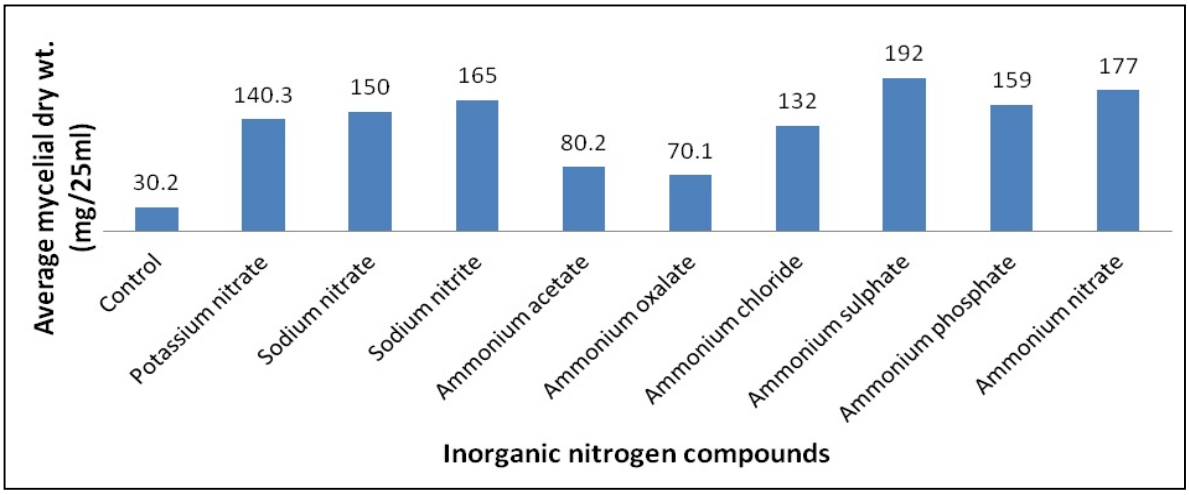

Figure 6: Growth and sclerotial production of *Morchella hybrida* with different inorganic nitrogeneous compounds at temperature 24°C, pH 7.0 and after ten days of incubation.

Table 7: Growth and sclerotial production of *Morchella hybrida* with different organic nitrogenous compounds at temperature 24˚C, pH 7.0 and after ten days of incubation.

Organic nitrogen compounds	Final pH	Average mycelial dry wt. (mg/25ml)	Sclerotial production
Control	7.11	10.0	-
L-arganine	7.01	52.2	+
L-cysteine HCl	6.81	38.0	-
DL-aspartic acid	6.79	45.5	-
L-cysteine	7.10	20.2	-
L-glutamic acid	6.92	102.0	+++
glycine	6.85	88.2	++
L-histidine HCl	7.0	50.0	-
L-leucine	6.72	72.2	-
DL-methionine	6.91	63.0	-
L-ornithine HCl	6.62	48.2	+++
L-β-phenylalanine	6.88	55.0	-
L-proline	6.92	35.0	-
DL-serine	6.71	32.0	-
L-tyrosine	6.99	40.0	-
DL-valine	6.81	58.0	-
DL-tryptophan	7.03	33.2	-
DL-threonine	6.95	70.1	-
Replicates: Insignificant, Variables: Significant, SE: 1.50, SE_d: 2.0, CD(5%): 4.36			

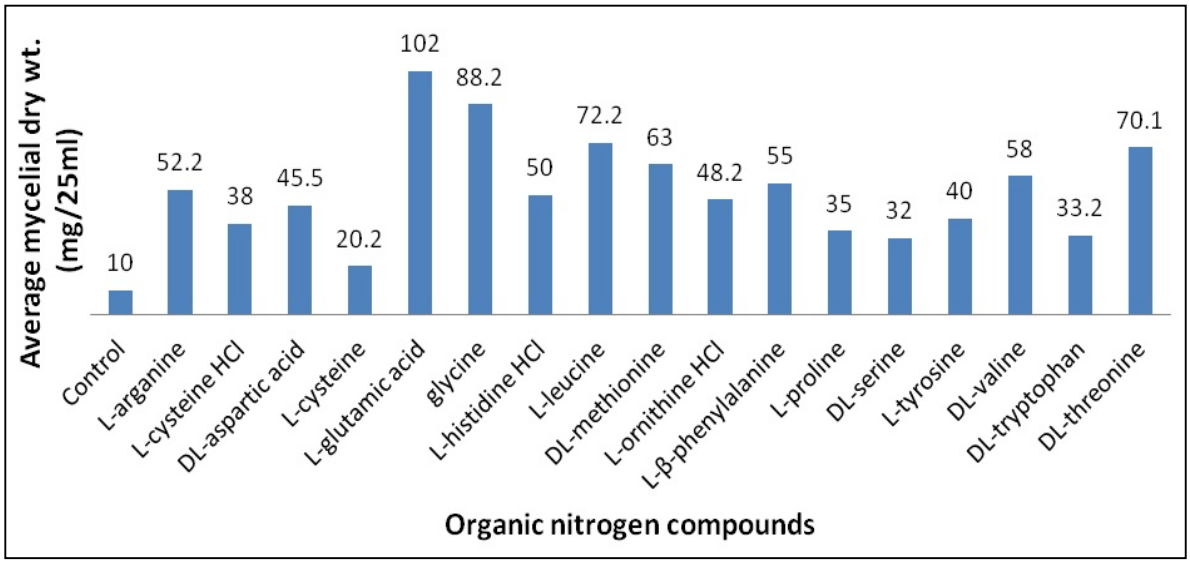

Figure 7: Growth and sclerotial production of *Morchella hybrida* with different organic nitrogenous compounds at temperature 24˚C, pH 7.0 and after ten days of incubation.

Organic Nitrogenous compounds: Seventeen organic nitrogen compounds were tested for the growth and sclerotial production. Sodium nitrate of the basal medium was substituted singly with each inorganic nitrogen compounds so as to provide 0.250 g of nitrogen, an amount present in 1.5 g of $NaNo_3$. Three replicates were kept for each nitrogen compound. The data on mycelial growth, sclerotial production and mean final pH of the cultural filtrate has been recorded after ten days of incubation. The statistical analysis

of the data on growth is appended to Table 7 and Figure 7. The fungus produced maximum mycelial growth with L-glutamic acid followed by glycine > L-leucine > DL-threonine > DL-methionine > DL-valine > L-β-phenylalanine > L-arganine > L-histidine HCl > L-ornithine HCl > DL-aspartic acid > L-tyrosine > L-cysteine HCl > L-proline > DL-tryptophan > DL-serine > L-cysteine. The fungus gave best production of sclerotia with L-glutamic acid and L-ornithine HCl whereas good production of sclerotia with glycine was observed.

CONCLUSION: The present study has found the effects of different carbon and nitrogen sources on the growth and sclerotial production of *M. hybrida*. The fungus grew best on Czapek's medium at optimum temperature 24˚C, pH 7.0 and ten days of incubation. Different carbon sources were tested but D(+)xylose was found to be best for mycelial growth and sclerotia production. L(-) sorbose and lactose showed poor growth and production of sclerotia. Various inorganic and organic nitrogen compounds were tested and the fungus produced best growth and sclerotia with ammonium sulphate.

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