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NUTRITIONAL STUDIES ON SPECIES AND MUTANTS OF LEPISTA, CANTHARELLUS, PLEUROTUS AND VOLVARIELLA

by

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ABSTRACT

The purpose of this study is to determine the ability of select Agaricales species to utilize various sources of carbon, nitrogen, vitamins, and growth hormones. Fungi selected for the studies include: *Cantharellus clavatus* FRIES, *C. cibarius* FRIES, *Lepista nuda* (BULL. EX. FRIES) COOKE, *Pleurotus ostreatus* (JACQ. EX. FRIES) KUMMER, and *Volvariella volvacea* (BULL. EX. FRIES) SINGER. Three strains of *C. cibarius* and one mutant of *V. volvacea* (V135), V134, were employed to determine if nutritional requirement differences occurred. One species, *V. volvacea*, is grown commercially as a cottage industry in the Orient (ALICBUSAN & ELA, 1961) while the other species currently have no commercial value. All species studied possess pleasing flavors and have potential use in the mushroom production industry.

A literature compilation of the nutritional regulation of basidiocarp formation and vegetative growth of Agaricales was made with specific mention to the named species (VOLZ & BENEKE, 1969). Recent nutritional studies with one or more of the specific species include those by YUSEF & ALLAM (1967), and EGER (1970).

MATERIALS AND METHODS

The homobasidiomycetes used in this study were collected from nature. Initial transfers of hyphae to agar plates were made aseptically from pileus tissue except for spore isolations of *Pleurotus ostreatus*, and hyphal stock cultures of *Volvariella volvacea* obtained from the University of the Philippines. Stocks were maintained on 2 % malt agar. Mutant strains of the selected species were isolated from agar plates and were also retained in stock on malt agar. Microscopic examination of mutant hyphae revealed similar growth dynamics compared to parent colonies, however, phenotypic change in colony growth dynamics was observed.

Inocula of the selected fungi grew in stock plate cultures for three weeks, after which a 1.5 inch square section of the colony was removed and blended one minute with 55 ml distilled H_2O in a 350 ml Monel metal semi-micro Waring Blendor. Five ml of the blended mycelium was pipetted into 250 ml DeLong Culture Flasks

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Table 1. The Utilization of Monosaccharides

Organism	D-arabinose	DL-arabinose	L-arabinose	D-fructose	D-galactose	D-glucose	D-mannose	L-rhamnose	D-ribose	L-sorbose	D-xylose	Carbon Free Control
C. clavatus (C8)	21	22	31	20	36	30	35	36	35	38	30	14
C. cibarius (C15)	45	102	51	45	23	108	69	45	34	41	85	25
C. cibarius (C86)	24	26	27	21	45	30	28	34	32	28	37	19
C. cibarius (C96)	147	219	319	251	245	280	275	88	228	214	253	17
L. nuda (L133)	30	93	50	172	41	269	340	25	31	45	26	33
P. ostreatus (P51)	26	28	38	24	31	31	33	41	27	49	37	27
V. volvacea (V135)	33	33	41	29	32	43	43	57	34	52	39	29
V. volvacea (V134)	20	22	26	16	31	24	28	36	31	23	42	15

Dry mycelial weight in grams

containing 50 ml test medium and then placed on New Brunswick Gyrotary Shakers at 180 RPM. A modified Humfeld's basal medium (HUMFELD & SUGIHARA, 1952) was used for the shake cultures and substitutions were made according to the nutrients tested. Ingredients of Humfeld's medium were glucose, 8 g carbon; urea, 0.47 g nitrogen; KH_2PO_4, 0.87 g; $MgHPO_4$, 0.40 g; $CaCl_2.H_2O$, 0.37 g; H_2SO_4 (2N), 5.1 ml; trace element solution, 20.0 ml ($FeCl_3.6H_2O$, 0.5 g; $MnCl_2.4H_2O$, 0.36 g; $ZnCl_2$, 0.20 g; $CuSO_4$, 0.05 g; distilled H_2O to make one liter); 1,000 ml distilled H_2O. The pH was adjusted to 4.5 with NaOH(1N).

Using various carbohydrates, 8 g carbon per liter was substituted for glucose, and a nitrogen equivalent based on one g urea per liter, or 0.47 g nitrogen per liter was selected for the carbon or nitrogen test systems respectively. Replicate flasks of 3 to 5 were made of each test system and were incubated on the rotary shakers for 20 days each for the carbon sources and 14 days for the nitrogen sources. Data were recorded as gram weight of the dried mycelium. DL-malic acid was Seitz filtered and pipetted aseptically to the individual sterile rotary shaker culture flasks. Water filtrates of casein and xylan were used.

A series of studies included the growth of 4 fungal test organisms grown in contact with 10 vitamin sources. Replicates of 3 were made of each vitamin at 0.5 mg per liter of glucose casein hydrolysate with agar (HOROWITZ & BEADLE, 1943). The media con-

Table 2. The Utilization of Disaccharides and Polysaccharides

Organism	D-cellobiose	α lactose	β lactose	D-maltose	sucrose	raffinose	dextrin	glycogen	inulin	pectin	starch	xylan
C. clavatus (C8)	32	29	31	28	32	36	33	31	29	45	31	15
C. cibarius (C15)	97	28	34	32	52	21	54	52	36	81	56	42
C. cibarius (C86)	32	23	38	34	28	106	33	26	39	45	43	18
C. cibarius (C96)	216	167	54	264	274	250	228	99	36	52	275	71
L. nuda (L133)	332	58	52	95	55	107	50	47	45	151	71	188
P. ostreatus (P51)	51	26	45	32	30	28	30	63	30	59	44	39
V. volvacea (V135)	59	36	35	44	43	30	62	267	37	50	85	40
V. volvacea (V134)	28	22	33	36	33	19	26	28	28	36	33	14

Dry mycelial weight in grams

tained glucose, 25 g; casein (vitamin free), 2 g; $MgSO_4.7H_2O$, 0.5 g; KH_2PO_4, 1.0 g; fumaric acid, 1.32 g; Na_2CO_3. H_2O, 1.12 g; $FeSO_4$, 0.2 mg; $ZnSO_4$, 0.2 mg; $MnSO_4$, 0.1 mg; purified oxoid agar no. 3, 20 g; distilled H_2O, 1,000 ml. Hyphal growth was measured on the agar surface. Growth promoting substances were also added to agar media in another hyphal study.

Results

Utilization of carbon sources

The pH of Humfeld's medium was varied to determine the most acceptable level for the selected fungal cultures. At 20 days growth *Lepista* produced the greatest mycelial dry weight at 4.5 while at 10 days growth, dried weight was somewhat greater at a slightly higher pH. *Pleuroius* growth was best at pH 6.5 at 10 and 20 days while strains of *Volvariella* and *Cantharellus* grew best at pH 4.5 at each harvest date.

Experimental results in Table 1 present the utilization of eleven monosaccharides and the carbon free control. Hyphal growth in shake culture produced good pellet formation for several cultures having high dry weights with exception of *Cantharellus* strains with loose hyphal pellets. Occasionally a mycelial ring developed on the flask wall at media splash levels if good growth

Table 3. The Utilization of Organic Acids and Miscellaneous Carbon Sources

Organism	citric acid	galacturonic acid	DL-malic acid	oxalic acid	pyrogallic acid	resorcylic acid	succinic acid	tannic acid	D-tartaric acid	i-inositol	D-mannitol	pectinol A	D-sorbitol	D-glucosamine hydrochloride	fructose-1,6-diphosphate	casein
C. clavatus (C8)	23	33	18	20	20	23	44	11	29	42	37	109	30	42	191	57
C. cibarius (C15)	25	48	55	20	13	21	38	23	17	44	49	116	47	56	317	19
C. cibarius (C96)	22	44	12	18	19	21	36	10	18	39	42	126	35	49	183	45
C. cibarius (C96)	222	35	206	20	26	21	92	32	223	194	340	298	307	214	396	56
L. nuda (L133)	27	202	17	16	29	21	22	43	24	55	44	234	51	32	249	68
P. ostreatus (P51)	24	25	25	20	15	22	25	32	21	28	48	68	32	21	264	36
V. volvacea (V135)	25	33	34	23	14	21	27	14	18	38	49	121	50	46	293	69
V. volvacea (V134)	27	24	8	19	14	19	9	20	19	26	24	38	25	22	198	16

Dry mycelial weight in grams

occurred. Flasks with poor mycelial growth contained hyphae as individual strands somewhat branched. *Volvariella volvacea* submerged growth was quite distinct compared to other genera grown in shake culture. Regardless of the carbon source, one large loosely interwoven cellular mass composed of chlamydospores and hyphal remanents formed (VOLZ, 1969). The *Volvariella* mutant developed smaller masses of hyphae and fewer chlamydospores.

Disaccharides, one trisaccharide, and polysaccharides were substituted in Humfeld's medium as the carbohydrate source at 8 g carbon per liter (Table 2). *Cantharellus* showed moderate growth with most of the listed carbon sources with the trisaccharide raffinose registering a good growth gain. Strain C96 usually recorded the greatest growth gains of all cultures examined. A water soluble yellow pigment increased in concentration with increased growth rates of chanterelle hyphae. *Lepista nuda* pellet formation was greater in disaccharide media than in media containing monosaccharides, with D-glucose and D-mannose being the primary exceptions. Mycelial growth of *P. ostreatus* was slightly favored in D-cellobiose, beta lactose, glycogen, pectin, and starch. In comparison to *V. volvacea*, growths of its mutant were less with each respective carbon source. Glycogen was extremely beneficial for the *V. volvacea* parent strain while moderate growth occurred with other high molecular weight carbon sources in both strains. Xylan and raffinose were not utilized by the mutant V134.

Organic acids were substituted as the carbon source with results tabulated in Table 3. Good growth was obtained with D-ga-

Table 4. The Utilization of Amino Acids and Related Nitrogen Sources

Organism	DL-alanine	L-asparagine	L-aspartic acid	betaine hydrochloride	creatine	cysteine hydrochloride	L-cystine	L-glutamic acid	glycine	D-histidine	DL-isoleucine	DL-methionine	L-proline	DL-threonine	DL-tryptophane	DL-valine	peptone	Nitrogen Free Control
C. clavatus (C8)	21	19	15	27	27	25	17	18	25	16	24	20	51	19	27	22	119	21
C. cibarius (C15)	41	17	74	39	30	35	28	65	56	18	63	53	98	87	48	49	154	18
C. cibarius (C86)	25	15	18	23	39	25	18	15	37	14	36	20	54	20	33	25	138	27
C. cibarius (C96)	340	217	424	387	81	218	53	464	291	90	274	121	338	323	262	355	320	78
L. nuda (L133)	157	75	99	50	51	18	31	115	418	20	46	102	52	42	41	65	236	41
P. ostreatus (P51)	87	34	49	29	33	35	18	27	22	24	31	33	30	43	38	28	84	21
V. volvacea (V135)	49	80	36	29	34	44	19	47	43	25	51	35	68	28	31	45	195	39
V. volvacea (V134)	15	17	13	20	24	23	19	16	17	17	16	19	38	20	33	20	87	13

Dry mycelial weight in grams

lacturonic acid, tannic acid, but variations occurred with strains of *Cantharellus*. *Lepista*, *Pleurotus*, and *Volvariella* also grew well with the same carbon sources except for *V. volvacea* mutant that showed no growth in media containing DL-malic, pyrogallic, or succinic acid.

Miscellaneous carbon sources, including sugar alcohols, were also introduced to the selected strains (Table 3). *Cantharellus clavatus* and two of the three *C. cibarius* demonstrated equal ability to utilize the compounds. One strain of *C. cibarius* (C15) grew well with fructose-1,6-diphosphate, and C96 recorded the greatest growth gains with most carbon sources. Casein was poorly utilized. Pectinol A and fructose-1,6-diphosphate generally produced high mycelial dry weights with most test species.

The utilization of carbohydrates at various concentrations was investigated. Up to 75 g of selected carbohydrates per liter of Humfeld's medium were added. Mycelial production commonly leveled off at 50 g carbohydrate per liter and showed little variation between 50 and 75 g per liter. Growth of the fungal species and strains was allowed to continue in shake culture with glucose as the carbon source. Maximum growth was obtained at 20 days with species and strains of *Cantharellus*, *Pleurotus*, and *Volvariella*. A gradual growth increase at succeeding harvest dates was noted with *Lepista nuda* and the mutant strain of *V. volvacea*.

Utilization of nitrogen sources

Sixteen amino acids and peptone were added singly in equal concentrations of nitrogen per liter of Humfeld's medium. Each of the 8 fungal strains was allowed to grow 14 days before harvesting, and dry weights of the mycelium were made (Table 4).

Table 5. The Utilization of Ammonium and Nitrate Nitrogen Sources

Organism	ammonium bicarbonate	ammonium chloride	ammonium citrate	ammonium nitrate	ammonium sulfate	ammonium tartrate	calcium nitrate	cobaltous ammonium sulfate	cobaltous nitrate	cupric nitrate	magnesium nitrate	potassium nitrate	sodium nitrate	uranium nitrate	urea
C. clavatus (C8)	41	24	27	27	26	25	26	29	14	21	24	27	25	15	31
C. cibarius (C15)	43	55	75	55	62	65	25	29	16	24	22	21	26	11	57
C. cibarius (C86)	44	26	28	28	31	49	34	33	16	25	43	23	37	16	58
C. cibarius (C96)	45	323	313	267	330	352	376	344	377	323	370	325	331	30	295
L. nuda (L133)	291	36	20	48	37	78	34	25	16	20	53	40	43	12	286
P. ostreatus (P51)	48	46	82	38	39	53	39	33	21	27	30	31	43	15	91
V. volvacea (V135)	46	31	51	48	43	54	66	29	19	22	40	47	49	15	49
V. volvacea (V134)	47	22	24	15	12	26	26	28	18	22	22	24	29	16	26

Dry mycelial weight in grams

Terminal pH at the end of 14 days was between 3.5 and 4.5 for all cultures, with an initial pH of 4.5 for control cultures devoid of a nitrogen source. *Cantharellus clavatus* (C8) produced mycelial dry weights equal to control with most amino acids, moderate growth increases with L-proline and excellent growth with peptone. Strain C15 of *C. cibarius* exhibited good growth increments with the respective amino acids. Peptone supported the greatest growth with the C15 isolate followed by L-aspartic acid, L-proline, DL-threonine, with only D-histidine and L-asparagine equal to control, the lowest growth supporters. *Cantharellus cibarius* C86 strain obtained moderate hyphal growth with creatine, glycine, DL-isoleucine, L-proline, and DL-tryptophane while the best growth was obtained with peptone. Excellent mycelial growth for strain C96 resulted with most all amino acid nitrogen sources. Creatine and L-cystine supported little growth for *C. cibarius* C96 which produced loosely organized pellets in the media containing amino acids. The other *Cantharellus* strains in turn developed minute pellets and growth at approximately one log less than strain C96.

Glycine and peptone both produced excellent hyphal growth in submerged culture for *Lepista nuda* while *Pleurotus ostreatus* had good growth in media containing DL-alanine and peptone, and no growth with L-cystine, glycine or D-histidine. *Volvariella volvacea* V135 and mutant strain V134 both grew best in peptone while V135 also grew well in L-asparagine. Moderate growth for both strains resulted with cysteine hydrochloride, L-proline and DL-valine. Minimum growth equivalent to control devoid of a nitrogen source was obtained with L-aspartic acid, L-cystine, D-histidine and DL-methionine for both *Volvariella* strains. Hyphae of the

Table 6. The Utilization of Vitamins

Organism	p-amino-benzoic acid	ascorbic acid	biotin	calcium pantothenate	niacin	niacinamide	pyridoxine hydrochloride	riboflavin	rutin	thiamine	control
V. volvacea (V135)	86	88	76	67	78	65	80	80	72	75	65
L. nuda (L133)	20	16	19	23	19	17	16	17	19	19	17
C. cibarius (C86)	16	16	18	23	15	9	16	19	14	22	4
P. ostreatus (P51)	45	43	39	51	41	35	35	32	38	51	30

Hyphal growth in mm at 12 days

Volvariella cultures grew as submerged mycelial masses, not as pellets.

Several ammonium and nitrate compounds were selected for nitrogen utilization studies in Humfeld's medium by the selected fungal strains, and were compared to urea (Table 5). Nitrogen content for each medium was 0.47 g per liter, or the equivalent amount of nitrogen in one g urea. Compounds producing hyphal growth greater or equal to that of urea for the selected fungal strains included: *C. clavatus* C8 - ammonium bicarbonate; *C. cibarius* C15 — ammonium citrate, ammonium sulfate, ammonium tartrate; *C. cibarius* C96 — all compounds tested except for ammonium bicarbonate and uranium nitrate; *L. nuda* L133 — ammonuim bicarbonate; *P. ostreatus* P51 — ammonium citrate; *V. volvacea* V135 — all compounds except cobaltous nitrate, cupric nitrate, uranium nitrate; *V. volvacea* V134 — all compounds except ammonium nitrate, ammonium sulfate, cobaltous nitrate, uranium nitrate. The pH at the time of mycelial harvest remained at the initial 4.5 but increased somewhat with nitrogen compounds ammonium bicarbonate and urea.

Utilization of vitamins and growth promoting substances

Ten vitamin sources were each added in concentrations of 0.5 mg per liter to glucose casein hydrolysate with purified agar (Table 6). Four test fungi were selected for this study. The table presents growth of hyphae at the end of 12 days, however, measurements were also made at 2 day intervals. *Volvariella volvacea* V135 had growth differences during the first 2 days of incubation but then growth rates became equalized. P-amino benzoic acid, ascorbic acid, biotin, calcium pantothenate, niacin and niacinamide

media supported initial hyphal growth, during the first 2 days after inoculation, of about 5 to 8 times greater than control. Pyridoxine hydrochloride, riboflavin, rutin or thiamine media had hyphal growth rates equal to hyphae grown in the control medium without a vitamin source. Calcium pantothenate and niacinamide had a slightly reduced growth rate compared with other vitamins but equaled growth of hyphae in the control medium.

Hyphal growth of *L. nuda* L133 and *P. ostreatus* P51 showed no significant growth rate differences on any of the vitamin agar media when compared to the control culture. *Cantharellus cibarius* C86 had approximately similar hyphal growth for all vitamin media except control and niacinamide. Strain C86 appeared to be the only one of the 4 fungi selected for vitamin study that demonstrated a dependency upon vitamins for mycelial growth.

Five growth promoting substances; gibberellic acid, kinetin, 2,4-dichlorophenoxy acetic acid, indoleacetic acid, and indolebutyric acid, were each added in concentrations of 0.1%, 0.01 % and 0,001 % to 2 % malt oxoid agar no. 3 and then inoculated with *V. volvacea* C135, *L. nuda* L133, *C. cibarius* C86, and *P. ostreatus* P51. Hyphal measurements in mm were taken at intervals similar to those in the vitamin studies. Growth hormones at 0.1 % concentration killed the inoculum primarily due to the low pH, 0.01 % also limited growth compared to control media devoid of growth substances, with or without acetone. No growth differences were noted in the 2 control media, acetone was used as solvent for the hormones. At 0.001 % concentration, no hyphal growth differences were noted with *Lepista, Cantharellus* or *Pleurotus* in any of the growth hormone media as compared to controls. *Volvariella* had an increased growth with the hormones especially gibberellic acid. Growth readings in mm for *Volvariella* were as follows at 0.001 % hormone concentration at the second and fourth day of incubation respectively: gibberellic acid, 22, 34; 2,4-dichlorophenoxy acetic acid, 15, 22; indoleacetic acid, 19, 25; indolebutyric acid, 18, 23: and kinetin, 1.4, 9; compared to control with acetone, 9, 26; and without acetone, 9, 27. After 4 days incubation, hyphal growth on all hormone cultures simulated development of the control cultures and in 8 days covered the entire agar surface of the petri plates.

Discussion

Previous studies on the selected fungal species have included observations on nutritional requirements for fructifications and vegetative growth. Glucose, fructose, mannose or xylose produced greatest hyphal growth for species of *Pleurotus* (Koch, 1958; Tsao, 1963). Polysaccharides dextrin, starch, and xylan were also selected as good carbon sources for *Pleurotus* by Koch (1958), Tsao (1963), Yusef & Allam (1967). Cellulose or lignin was utilized

by *Tricholoma* (MELIN, 1946; NORKRANS, 1950; REUSSER, SPENCER & SALLANS, 1958) and *Pleurotus* (CAMPBELL, 1932; WOLPERT, 1924). Carbon sources for nutrient induced fruiting of *P.ostreatus* included corn steep liquor, molasses, glucose, cellulose, and peptone while starch was negative (KLIOUSHNIKOVA, et al, 1935; BLOCK, TSAO & HAN, 1959).

Yeast and malt extracts have been most commonly selected by authors to produce mycelial growth and fruit body formation with the select fungi in undefined media. YUSEF (1950) found the beneficial effect of malt extract was largely the result of thiamine and amino acids in the natural product. The extracts have been selected for *Tricholona* and *Pleurotus* FRIES, 1938; HUMPHREY & SIGGERS, 1933; YUSEF, 1950; MIKOLA, 1955).

Ammonium salts and nitrates produced moderate growth with *Pleurotus* compared to the better growth with peptone, urea and amino acids (BLOCK, TSAO & HAN, 1959; KOCH, 1958; LEONIAN & LILLY, 1938, 1940; STEINBERG, 1939; TSAO, 1936). L-asparagine, DL-glutamic acid, glycine, and leucine served as good nitrogen sources for *P. ostreatus* (YUSEF & ALLAM, 1967). Greatest hyphal growth was obtained with amino acids and urea while ammonium salts increased growth, nitrates were slightly or not utilized by species of *Tricholoma* (MELIN, 1953; NORKRANS, 1950, 1953; REUSSER SPENCER & SALLANS, 1958). Vinnasse, a fermentation product of sugar beets, high in ammonium salts was useful for *Tricholoma* but useless to *Cantharellus* (FALANGHE, 1962).

Vitamins have increased fruit body production of several Agaricales. Those affected and the vitamins involved include *Pleurotus ostreatus* and thiamine (BLOCK, TSAO & HAN, 1959; KOCH, 1958; KURANCOWA, 1963; TSAO, 1963), plus thiamine, inositol, and biotin for increased growth of *Tricholoma nudum* (KOGL & FRIES, 1937; STEINBERG, 1939). *Pleurotus ostreatus* had an increase in spore germination and hypal growth with 2,4-dichlorophenoxyacetic acid, a growth promoting substance (KURANCOWA, 1963).

Many diverse concoctions and environmental factors have been selected for study of fungal growth and development. A survey of previous studies in relation to fructification and mycelial growth of Agaricales with special reference to *Pleurotus, Volvariella, Cantharellus* and *Lepista* has been made (VOLZ & BENEKE, 1969). Select species and strains of the genera were subjected to additional carbon, nitrogen, vitamin and growth promoting substances for further nutritional evaluations. Variation in nutritional regulation of growth was found within strains of the same species and between related species presented in this study.

Summary

Select species and strains of edible mushrooms were grown in vitro for an evaluation of nutritional requirements. The species under study have pleasing flavors and present potential use for commercial production.

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