THE USE OF PLANT TISSUE CULTURE TECHNIQUES [1] FOR STUDYING THE GROWTH OF MOREL

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ABSTRACT

Sections of the stipe of the morel mushroom, *Morchella esculenta,* were explanted on modified Murashige and Skoog's medium (M&S). The subsequent culture was cloned and various concentrations and sources of nitrogen, potassium, phosphorus, sugar and growth factors were tested. Liquid shaker culture, agar culture, and peat pellets placed in liquid were the methods used for growing the organism in a constant temperature incubator. The subsequent growth was quantified and plotted over a period of time. The results indicate a preference for ammonium over nitrate nitrogen. The maximum growth occurred with 0.05 M phosphate and it showed good growth up to 0.6 M with potassium salts. It grew on many sources of sugar but preferred glucose, mannose, and sucrose. Morphological growth habits, such as sclerotia formation, were also observed and recorded. The original inoculum source, from the Elk Point Sand Dunes, was maintained in rye culture in the refrigerator for over two years. This was used when new cultures were needed.

INTRODUCTION

The Morel mushroom is the sporocarp produced by members of the genus *Morchella.* Mushroom hunters avidly search for morels throughout most of North America and Europe each spring (Singer 1961). Morels are found in late April through May in South Dakota. The exact date depends on temperature and moisture. Morels are found in grasslands and wooded areas. The Elk Point sand dunes in South Dakota are usually a good place to find morels. Morels are usually found on fertile, moist soil with abundant organic matter and a pH near 7 (Delmas and Bunel 1974). Morels are often found in unusually large numbers near plants that have been injured (Bartelli 1969, Baker and Matkin 1959). At one time people in Germany set fires in wooded areas to insure a morel crop the following spring (Christensen 1943). In more recent years mushroom hunters have found large numbers of morels near diseased elm trees (Bartelli 1969).

Morels have been reported in unusual places such as a mine (Moser 1949), a greenhouse in California (Baker and Matkin 1959), and on a bench where ferns were grown in a florist shop.

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The florist shop where morels occurred is in Lafeyette, Indiana. The morels began appearing in early February 1973, about two months before the usual date of appearance. Most of the morels were small, some were as small as a pencil point, but one was 6 inches tall. The morels came up in pots with ferns and in gravel which covered the bench. The gravel had been added in December. The bench was located in a basement where the temperature was 75 F during the day and 65 F at night. The bench was constantly illuminated by flourescent lights. Although the florist is not aware of any changes, no morels have appeared since March, 1973 (personal communication with Mabel Roth, florist).

Information on the life cycle of *Morchella* is incomplete. The requirements for initiation of reproductive growth are unknown. Some people have obtained sporocarps by enhancing natural conditions by adding compost, apple mash, leaves, twigs, and nitrogen fertilizer to the soil (Brock 1951, Cailleux 1969, Singer 1961). A reliable method has never been developed. Sporocarps have never been reported in pure culture (Brock 1951).

Morchella is not difficult to grow in the vegetative state. *Morchella* can utilize proteins, amino acids, ammonium or nitrate salts as nitrogen sources (Brock 1951, Litchfield 1967). Brock reports that nitrate is utilized if the pH is near neutral. Most simple or complex carbohydrates will fulfill requirements for carbon and energy. Starch, maltose, glucose, fructose, and sucrose are some of the better sources (Brock 1951, Litchfield 1967). The principle minerals required are S, P, K, Mg, and Fe (Williams et al. 1956). Mn and Ca improve the ability to withstand an acid environment (Robbins and Hervey 1965). Most reports say *Morchella* has the best growth when the pH is near neutral (Brock 1951, Litchfield 1967, Williams et al. 1956, Robbins and Hervey 1965). Williams et al. (1956) found no response to the presence of the vitamins pantothenic acid, biotin, thiamine, or pyridoxine. Hurni (1946) reported *Morchella* was slightly inhibited when thiamine was added to the media. *Morchella* is not affected by light (Williams et al. 1956).

The purpose of this study was to observe growth response to nutritional factors especially noting responses indicating the initiation of reproductive growth.

MATERIALS AND METHODS

Cultures were grown in agar, peat, and in submerged liquid media. The medium (modified MS) was made up from Murashige and Skoog's salts (Murashige and Skoog 1962) with 0.05 M phosphate buffer, 30 g sucrose/1, and a pH of 5.6. Media was sterilized by autoclaving 15 minutes at 15 pounds pressure and at 120 C. Vitamins were sterilized by filtration.

The inoculum originated as stipe tissue from a *Morchella* sporocarp collected at the Elk Point sand dunes. Inoculum was maintained on rye and on agar plates. Rye cultures were refrigerated at approximately 5 C and served as a reserve. Cultures on agar plates were used to inoculate media in the tests. Potato dextrose agar was used to grow the inoculum cultures except for the tests with vitamins. Media in vitamin tests was inoculated from the second subculture on modified medium.

The response of *Morchella* was evaluated according to the final dry weight and the form of growth. Dry weights were taken after drying approximately 8 hours at 100 C.

Cultures on agar were used to study the response to variable source and concentration of nitrogen and carbohydrates, concentration of potassium and phosphorus, and pH. Culture vessels were 125 ml Erlenmeyer flasks, with 50 ml of media. Agar cultures were incubated 21 days at 25 C.

Cultures on peat pellets (Jiffy Seven, Jiffy Pot Ltd., Grorud, Norway) were used to study the effect of varying the nutrient availability. Peat cultures utilized peat pellets placed in 600 ml beakers with 50 ml media. A pellet is a cylinder of peat 4 cm high by 4 cm in diameter, held together by fiberglass cloth. Cultures were transferred to fresh media twice a week. The composition of the media was changed slightly each time. Cultures receiving variable nutrient solutions started out at 0.1x modified M&S medium. At 2.5 weeks the cultures were on 2x media. From the third through the fifth week the concentration was gradually reduced until the cultures were on deionized water the fifth week. Similar experiments were carried out changing the concentration of nitrogen and the concentration of sucrose. The concentration of nitrogen (NH_4NO_3) began at 0.005 M, rose to 0.02 M, and then was finally reduced to 0 at the beginning of the fifth week. The concentration of sucrose began at 5 g/1, rose to 100 g/1, and then was reduced to 0 on the fifth week. A group of cultures in each experiment was kept at 25 C and another was kept at 17 C. The control cultures for these experiments received the same total quantity of the variable nutrients, but in equal amounts with each transfer. Cultures were kept 3 weeks after being transferred the final time.

Submerged cultures served to determine the growth with additions of vitamins to the media. The culture vessels were 125 ml Erlenmeyer flasks with 40 ml medium. Cultures were allowed to grow five days at room temperature with constant shaking at 100 rpm on a reciprocating shaker.

RESULTS AND DISCUSSION

Results are the mean of five replications. In some cases there were fewer replications due to contaminating organisms.

Analysis of variance indicates that differences in growth due to treatment effects exist, when tested at the 0.01 level for all experiments.

The effect of four sources of nitrogen at five levels was tested.

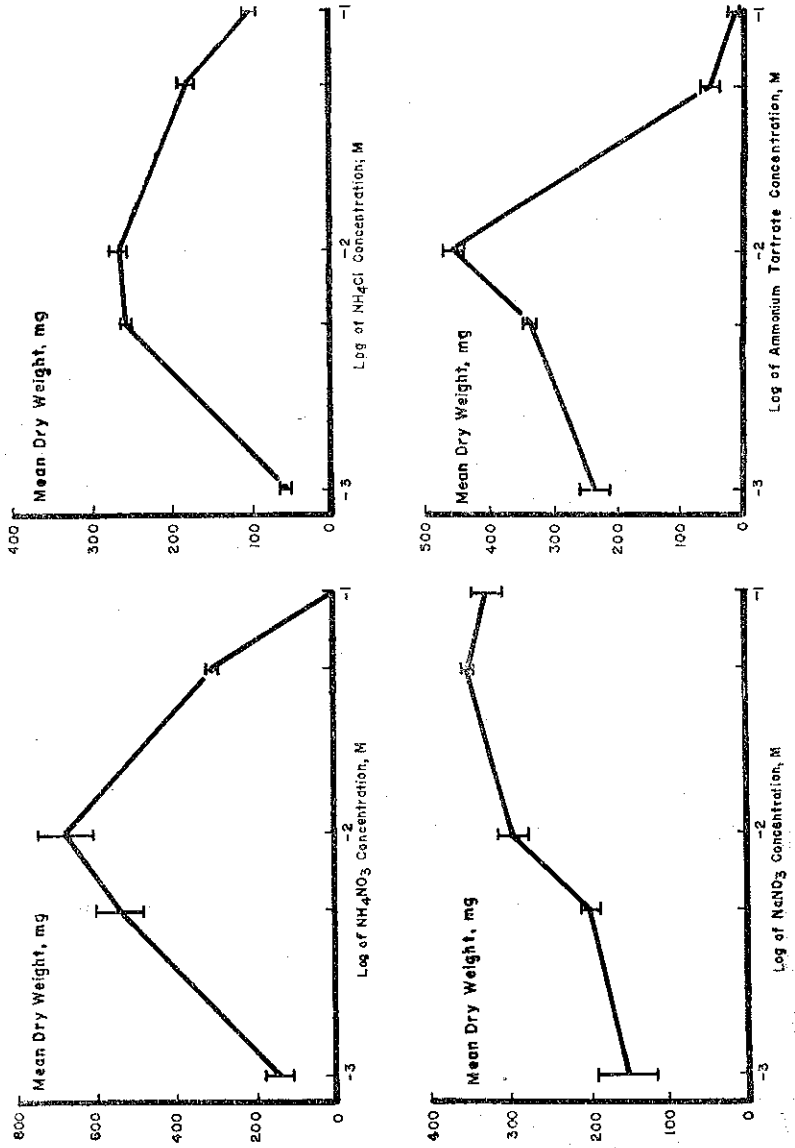

Figure 1. Growth by Morchella on four nitrogen sources.

The sources NH_4NO_3, ammonium tartrate, NH_4Cl, and $NaNO_3$, were tested at increasing concentration. The results are summarized in Figure 1. Note that growth on all NH_4 salts showed the same trend, with the greatest growth at 0.01 M and inhibition at higher levels. No cultures grew on 0.1 M NH_4NO_3. The mean dry weight was 666 mg on 0.01 M NH_4NO_3, 454 mg at 0.01 ammonium tartrate and 297 mg on 0.01 M NH_4Cl. The response to the level of $NaNO_3$ was different. The greatest amount of growth, 348 mg, occurred at a concentration of 0.05 M. There was little or no inhibitation at 0.01 M $NaNO_3$. When NH_4NO_3 was the nitrogen source on unbuffered medium the final pH was 3, or less. This indicates preferential uptake of NH_4 when both NH_4 and NO_3 are present.

The effect of variable levels of potassium (Figure 2) was studied using KNO_3 as the potassium source at concentrations from 0.01 to 0.6 M. Ammonium phosphate (monobasic) was substituted at 0.05 M for the usual nitrogen sources. The cultures responded positively to increasing levels of KNO_3 up to 0.25 M where the mean dry weight was 654.4 mg. There was little difference among the cultures at higher treatment levels, with no inhibition observed at 0.6 M. The greatest amount of growth was 687 mg at 0.5 M.

The effect of different levels of phosphate was tested over a range from 0.001 to 0.2 M (Figure 3). Phosphate was supplied as a buffer made up of Na_2HPO_4 + KH_2PO_4. Figure 3 summarizes the

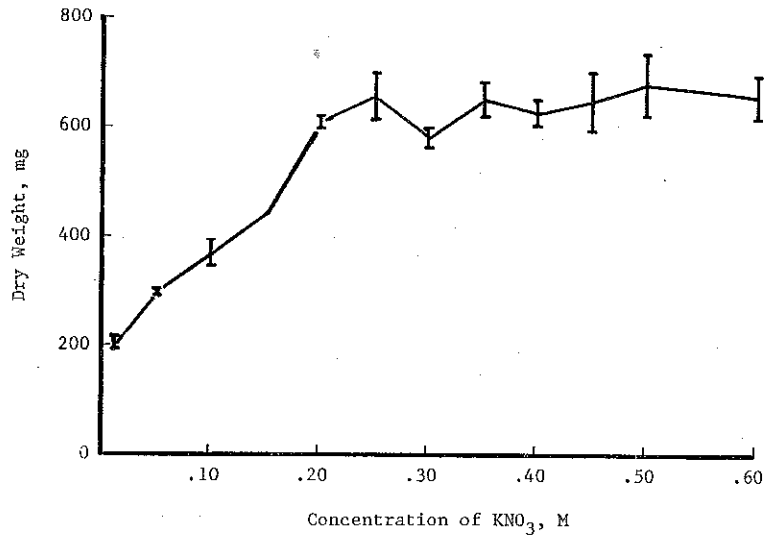

Figure 2. Growth of Morchella with different concentrations of KNO_3.

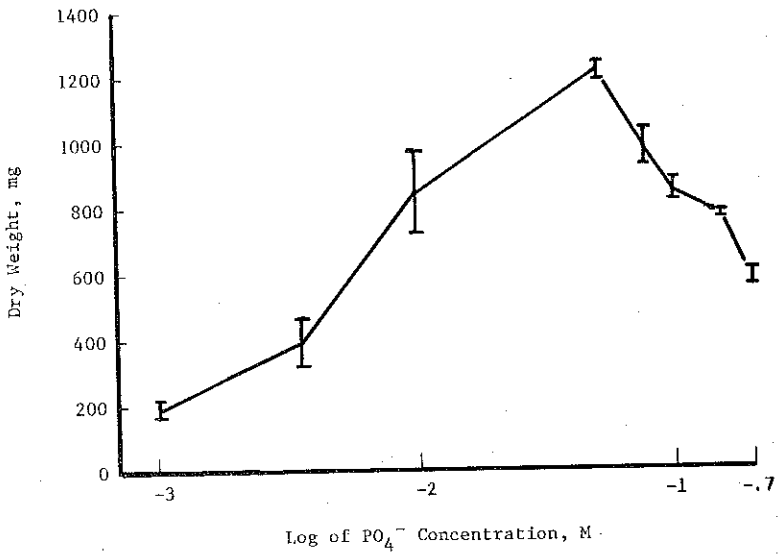

Figure 3. Growth of Morchella at different levels of PO_4.

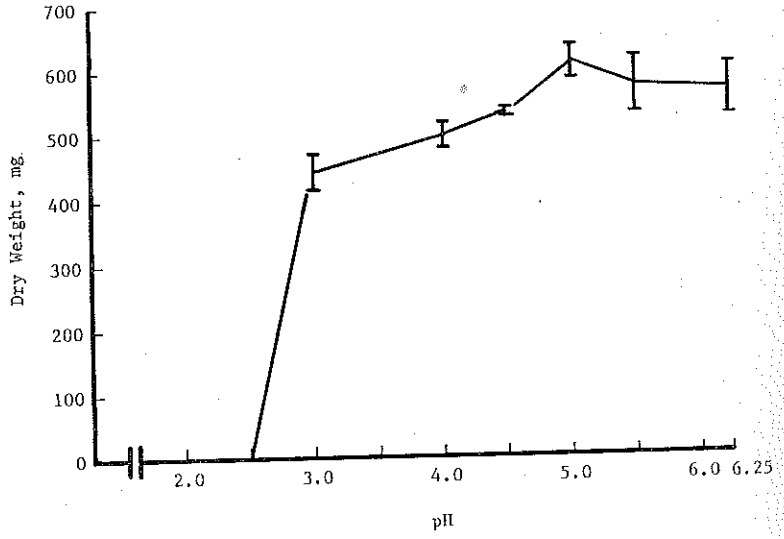

Figure 4. Growth of Morchella at variable initial pH.

results. The highest dry weight, 1223 mg, was found when the phosphate concentration was 0.05 M. Growth was reduced at higher levels. Buffering capacity of the phosphate salts is probably a factor in the response to levels of phosphate from 0.001 to 0.05 M. The final pH at 0.001 M was 3; at 0.01 M, 3.5; and at 0.05 M the final pH was 5.0.

The initial pH was varied by using different levels of citric acid from 0 to 0.215 M with a 0.05 M phosphate buffer. Ammonium tartrate at 0.01 M was used as the nitrogen source. The results are summarized in Figure 4. Cultures grew well over the range from 3 to 6.25. The greatest amount of growth, 612 mg, was found when the initial pH was 5.0. Most researchers have reported a neutral pH to be optimum. Differences could be due to genetic differences, differences in nitrogen source, or differences in the minerals included in the media. Robbins and Hervey (1965) found that the level of Mn and Ca is an important factor for acid tolerance by *Morchella*. Other researchers (Brock 1951, Litchfield 1967) did not use Mn or Ca in their medium.

Different kinds of carbohydrates were tested with M&S medium and modified M&S. In the modified medium the ammonium nitrate was 0.03 M, KNO_3 was 0.3 M, and PO_4 was present at 0.05 M. Monosaccharides were supplied at 0.2 M and disaccharides at 0.1 M. The results are summarized in Table 1. Glucose, mannose, and sucrose are the best sugars with both nutrient salt solutions.

TABLE 1

Growth With Different Carbohydrates and With Two Nutrient Salt Solutions*

	M&S Salts		M&S (+High NPK)	
Carbohydrate	Mean, mg	S. D.	Mean, mg	S. D.
Glucose	606.60	90.45	626.40	74.71
Mannose	587.80	60.83	755.00	50.43
Sucrose	337.60	96.54	674.20	46.00
Galactose	224.00	53.80	391.40	55.80
Lactose	162.00	56.62	185.76	22.60
Rhamnose	156.60	6.58	77.60	15.77
Fructose	121.60	14.17	592.00	71.68
Arabinose	109.20	28.54	207.40	54.11
Gluconic acid	69.40	20.42	15.33	0.58
Xylose	68.75	7.89	332.60	61.24
Galacturonic acid	35.00	2.83	15.50	7.78

*Measured on a dry weight basis.

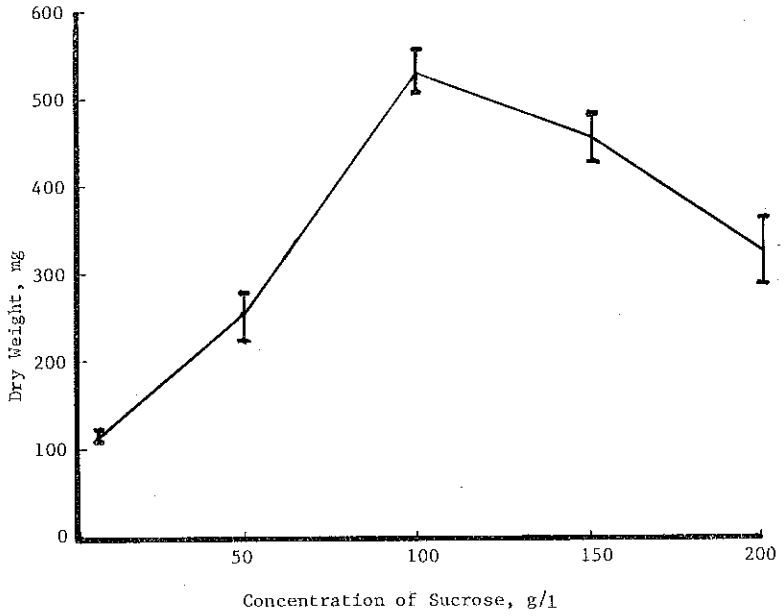

Figure 5. Growth of Morchella with different concentrations of sucrose.

The effect of increasing concentration of carbohydrate was tested using sucrose at levels from 5 to 200 g/1. Cultures with 100 g sucrose/1 had the most growth, 527 mg. Figure 5 summarizes the results.

Cultures on peat were evaluated according to morphology only. There was no difference in the form of growth, regardless of the treatment. All cultures developed a large mass of sclerotia at the top with mycelium spreading out across the surface of the medium.

There were 11 treatments in the vitamin study. Nine treatments had a single vitamin or inositol. One treatment included all eight vitamins and inositol. The control had no vitamins. The results are summarized in Table 2. Little growth was noticed until the last day in medium containing nicotinic acid, thiamine, or inositol. Cultures containing other vitamins had a wide range in response, within treatments, compared to the control. Dunnett's test at 0.05 level indicates no differences due to treatment effects when comparing the control to other treatments. These results are similar to those obtained by Williams et al. (1956) and Hurni (1946).

Most cultures developed a mat of mycelium, which was often quite thick. The only other form of growth was the development

TABLE 2

Growth With Different Vitamins Added to the Media*

Vitamin Added	Concentration, μg/1	Mean, mg	S. D.
Nicotinic acid	400	0.14	0.31
Thiamine	400	0.52	0.75
Inositol	2000	5.56	5.19
Biotin	2	29.38	17.66
ρ-aba	200	40.14	15.25
Pyridoxine HCl	400	49.28	14.84
Ca pantothenate	400	51.14	9.74
Riboflavin	200	42.74	14.93
Folic acid	2	46.30	16.23
All of the above	As indicated above	32.88	19.85
None	.....	31.84	5.46

*Measured as dry weight.

of sclerotia. *Morchella* is known to produce sclerotia, but their function is unknown (Cailleux 1969, Brock 1951). In the present work it was found that sclerotia are seldom produced if NH_4 is present, but are produced in large numbers if NO_3 is the sole nitrogen source. When the level of KNO_3 varies from 0.02 to 0.06 M, the number of sclerotia is directly related to the KNO_3 concentration, while size is inversely related. Sclerotia were rarely produced if the concentration of phosphate was greater than 0.01 M.

CONCLUSIONS

1. The tested nutritional factors are not of primary importance in the initiation of reproductive growth.
2. *Morchella* utilizes NH_4 or NO_3 salts as nitrogen source, but utilizes NH_4 more rapidly.
3. *Morchella* utilizes levels of KNO_3 as high as 0.25 M and is not inhibited by levels as high as 0.6 M.
4. The optimum level of the phosphate salts is 0.05 M with inhibition at higher levels.
5. *Morchella* grows well in the pH range of 3 to 6.25.
6. *Morchella* can grow well with most sugars but glucose, mannose, and sucrose are often utilized best.
7. *Morchella* effectively utilizes levels of sucrose as high as 100 g/1, with reduced growth at higher concentration.
8. Sclerotia are produced in large numbers with NO_3 as the sole nitrogen source if the concentration of PO_4 is not greater than 0.01 M.
9. *Morchella* has little or no requirement for exogenous vitamin supplies.

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