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The Use of Plant Tissue Culture Techniques for Studying the Growth of Morel

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THE USE OF PLANT TISSUE CULTURE TECHNIQUES FOR STUDYING THE GROWTH OF MOREL

BY

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THE USE OF PLANT TISSUE CULTURE TECHNIQUES

FOR STUDYING THE GROWTH OF MOREL

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

_____ Thesis Advisor Date

_____

Head, Botany-Biology Department Date

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INTRODUCTION

Like all mushrooms, the morel mushroom is a fungus. Morels are the fruiting bodies produced by members of the genus, Morchella. Morels are often considered to be in a class with truffles by mushroom hunters.

Unlike other mushrooms, the morel is not toadstool shaped, but looks more like a sponge on a stalk. This makes it easy to identify. There are no poisonous mushrooms that resemble morels.

Morels are widely distributed. Morels are known to occur in North America, Europe, and Israel. They are found in a wide variety of topographies, from mountains to plains. They may be found on grasslands, orchards, and forests.

The time of fruiting is almost always in the spring. Morels have also been found in the summer and fall, but this is rare. Fruiting of Morchella is unpredictable. Sporocarps may be found in large numbers one year and not reappear in the same area for many years.

In spite of widespread popularity, Morchella has never been grown to the sporocarp stage in pure culture. Like other mushrooms, morels are sources of proteins and B-vitamins.

The purpose of this study has been to gather information on the physiology of Morchella. The response to various nutritional factors was evaluated in terms of amount of growth, and morphogenetic responses. It was hoped that by varying different factors of the medium, it would be possible to find the factors leading to sporocarp formation.

LITERATURE REVIEW

The morel mushroom has been studied for almost 100 years. Ecological studies, studies of growth in pure culture, and studies of growth on natural materials such as compost, have been performed.

The ultimate goal of most researchers has been the study of the stimulus for sporocarp formation. There are some reports of success in obtaining sporocarps using natural materials in non-sterile conditions, but a repeatable method using these techniques has apparently not been found.

Researchers have studied nutritional requirements, as well as the effects of variable environmental stimuli. A sporocarp has never been obtained in pure culture. However, it has been found that the mycelium may be successfully and economically grown on a commercial scale for use in human foods.

Information on the life cycle of Morchella is incomplete. It is known that sclerotia may be formed. Some workers report that a conidial stage exists. It is not known whether the formation of sclerotia or conidia precede sexual reproduction, or if they are detours from the path to sporocarp formation.

Spores and Spore Germination

Spores of Morchella will germinate as soon as they are provided with favorable moisture and temperature conditions. Falck (1920) found that spores would germinate in a petri dish containing agar or gelatin. Upon germination, a spore of Morchella may give rise to more than one hyphae (Falck, 1920; William and William-Engels, 1956).

Sclerotia

Sclerotia are sometimes formed by the Morchella mycelium (Singer, 1961; Cailleux, 1969; Baker and Matkin, 1959; Sanderson, 1969). The function of these sclerotia has not been determined. Conrad (1958) stated sclerotia supply nutrients for developing Morchella sporocarps. Falck (1920) suggested that Morchella sporocarps may arise from sclerotia.

Generally speaking, sclerotia may be involved in the development of fruiting bodies, or they may function as structures for vegetative reproduction and survival (Ainsworth, 1966; Cochrane, 1958; Townsend and Willetts, 1954; Hawker, 1957). Sclerotia are resistant to unfavorable conditions and may remain viable in corked test tubes from two months to more than six years (Ainsworth, 1965; Hawker, 1950). Sclerotia contain storage materials necessary for the resumption of growth in favorable conditions (Ainsworth, 1966; Hawker, 1950; Townsend and Willetts, 1954). Molliard has reported growing sclerotia of Morchella on sterile, moist bread. They were large and resembled sporocarps in appearance and taste, but they never formed asci (Brock, 1951).

Conidia

Constantin (1936), Cailleux (1969), and Molliard (Brock, 1951) have reported a condidial stage of Morchella. This conidial stage is usually classified as a Fungi Imperfecti, and called Constantinella cristata. Cailleux (1969) has tried numerous treatments in order to germinate the conidia, but without success.

Stages of Sexual Reproduction

Information on sexual reproduction by Morchella is limited. Plasmogamy occurs shortly before ascus development (Singer, 1961; Gries, 1940). Gries (1940) studied fruiting bodies of Morchella in various stages of development. He found that plasmogamy was accomplished by fusion of vegetative hyphae. Gries (1940) found that plasmogamy occurred in the subhymenium, or just below it. The vegetative hyphae fuse, giving rise to a binucleate cell, that has one nucleus from each cell. This cell may become the ascus, or short ascogenous hyphae may be produced. The end cell becomes the ascus. Hook formation, common to ascomycetes, does not occur. Gries (1940) found that elongation of the stipe followed plasmogamy. Falck (1920) stated that growth in preparation for sporocarp formation occurs near the soil surface.

Geographical Distribution

Morchella is found in many countries and on different continents. It is common throughout Europe and North America (Singer, 1961; Conrad, 1958). Morchella is found in certain parts of Mexico and Israel (Rodriguez and Herrera, 1962; Nemlich and Hershenzon, 1972).

Habitat

Morels are not limited to any particular topography. They may be found on mountains, hills, or plains (Delmas and Bunel, 1974). Sandy soils may be better than finer texture soils (Frieden, 1969). In Israel, morels were found on sandy loam soil, rich in humus (Nemlich and Herschenzon, 1972).

Soil moisture is an important factor in relation to sporocarp production by Morchella. The soil must be moist, but not saturated. Dry soil does not favor fruiting of Morchella (Delmas and Bunel, 1974). Sanderson (1969) measured moisture content of soils where sporocarps were found. He found that soil water content (w/w) averaged 31.9% at a depth of 1 inch and 23.3% at 4 inches.

Morchella is more likely to fruit on soils having high organic matter contents (Delmas and Bunel, 1974). Sanderson (1969) found that soil organic matter ranged from 7.2% to 20.1% at four locations in South Dakota. Sanderson (1969) found that mycelium from which sporocarps arose appeared to be more often associated with living than dead materials.

Delmas and Bunel (1974) measured the C:N ratio where morels were found. They found the C:N ratio to range from approximately 10 to 20. They also found that morels were found on soils that were high in potassium and phosphorus.

Morels are usually found where the soil pH is near neutral. Sanderson (1969) found that soil pH ranged from 6.5 to 6.7 at a depth of 1 inch, and from 6.9 to 7.05 at 4 inches. Grainger (1946) found that morels had disappeared from an area near Halifax, Scotland after a period of over 150 years. During this period the soil pH had changed from neutral to acidic. Delmas and Bunel (1974) found Morchella sporocarps on soil with pH ranging from 5.5 to 8.0. Different species were associated with different pH ranges.

Season

Morels are usually found in the spring. The exact date depends on the climate. Morels appear from February, in the Southern states, to

early June in Michigan (Conrad, 1958; Bartelli, 1969). The fruiting season lasts two to four weeks (Sanderson, 1969; Alexopoulus, 1962). In 1967 and 1968 Sanderson (1969) found morels from late April, at Yankton, South Dakota through late May, at Pierre, South Dakota.

Delmas and Bunel (1974) reported that morels appeared when temperatures ranged from 8 to 16°C. Sanderson (1969) measured soil temperatures where morels were found. At the 1 inch to 2 inch depth, temperatures ranged from 6 to 17°C.

Morchella is known to fruit at other times of the year, but this is rare. Krieger (1967) and Frieden (1969) say it is rarely found in the fall. Morels have been found in October in Michigan (Bartelli, 1969). Morels were reported in August, at Yankton, South Dakota (Sanderson, 1969). Moser (1949) reported finding large numbers of morels from May through the summer and into September of 1948. He also reported a single morel in November.

Associated Plants

Morels are found in association with a wide variety of higher plants. They are found both in coniferous and deciduous forests, as well as grasslands (Christensen, 1943; Krieger, 1969; Frieden, 1969). Delmas and Bunel (1974) reported that 48% were in thin woods and brush, 21% in open terrain, 12% in clearings in woods, and 8% were in thick woods. Untended orchards are especially good producers of morels (Christensen, 1945). Morels are often found near Jerusalem artichokes (Baker and Matkin, 1959). Roze reported finding Morchella parasitizing rhizomes of Jerusalem artichoke (Brock, 1951).

It is not known what relationship Morchella has with higher plants. Repin, in 1901, established that Morchella is able to exist saprophytically (Brock, 1951). Singer (1961) has suggested that Morchella needs to grow parasitically to enter the reproductive phase. Singer (1961) has also suggested that Morchella may be a facultative mycorrhizae. Sanderson (1969) described Morchella mycelium concentrated around living roots, but not as a mycorrhizal form.

Conditions Favorable to Fruiting

Morels are more likely to be found near plants that have been injured. Morels are often found near dead or dying elm trees, Figure 1 (Bartelli, 1969). They are often found where trees have been cut down (Bartelli, 1969; Sanderson, 1969).

Morchella commonly fruits in large numbers the spring following a fire (McIlvaine, 1902; Christensen, 1943; Baker and Matkin, 1959). Fires seem to cause changes that are likely to promote fruiting by Morchella. At one time, people in Germany would deliberately set fires to insure a crop of morels the next spring. A royal decree was eventually issued to protect the forests (Christensen, 1943; McIlvaine, 1902). McCubbin reported "immense numbers of morels" in an area that had been burned over the previous October (Baker and Matkin, 1959). The morels came up the following spring. McCubbin found the morels up to the edge of, but not beyond the burned area. Smith (1963) reported finding "stupendous quantities in the wake of forest fires."

Moser (1949) has reported an interesting instance where morels came up following a forest fire. The fire was in August, 1947 and was

unusually intense, completely destroying all vegetation. In May, 1948 the morels began to appear. The morels were unusually numerous and unusually large, some were 20 to 25 cm tall and one was 30 cm tall. Most of the sporocarps came up near burned roots, branches, and stumps. The sporocarps continued to be produced as late as November, 1948.

Unusual Occurrences

In a flower shop basement in Lafayette, Indiana, morels came up on a bench where ferns were being grown, Figure 2. The morels appeared in early February, 1973, about two months before morels normally appear in Indiana. Most of the morels were small, some as small as a pencil point, but one was 6 inches tall. The largest one came up in a pot with a fern plant. Mycelium could be seen growing through holes in the bottom of the pot. Other morels were in pots with ferns or out in the gravel that covered the bench.

The bench was located in a basement. The bench was lined with plastic and covered with washed pea gravel. The pots were set down in the gravel. The soil in the pots came from Florida. The soil was high in organic matter and consisted mainly of peat moss. The florist had not added anything but water to the soil.

Temperature was goverened by a furnace in the basement. The thermostat was set at 75^{o} F days and 65^{o} F nights. The area was constantly illuminated by flourescent lights.

Gravel had not been used before December. Before adding gravel the pots had often dried out and the plants were often killed. The soil was always moist after the gravel had been added.

Figure 1. Morels that were found near the base of a dead elm tree. A large root was found directly beneath the morels. Date of occurrence was May 10, 1976. Note unusual abundance and several sporocarps seem to be fused together. 1/4 X.

Figure 2. Morels found in the flower shop basement at Lafayette, Ind. The morel in the pot on the right has deliquesed. These occurred early February, 1973. 1/4 X.

Although the florist is not aware of any change in conditions, morels have not reappeared since March, 1973 (personal communication with Mable Roth, florist).

Baker and Matkin (1959) reported an unusual occurrence in Cymbidium beds. Morels began appearing in February, 1956. This was during a cool period with a few light rains. The location was about 20 miles west of Santa Monica, California. The benches had been filled in July to September, 1955 with ground bark of white fir. The logs came from an area which had been burned in 1955 and logged soon after. The bark was fumigated with methyl bromide. Dolomite lime was added.

The benches were then planted with Cymbidium divisions. A solution of ammonium nitrate plus potassium chloride was applied at one to two week intervals. At the time when sporocarps appeared the bark was covered with mycelium. Morels have not appeared since this time. The only unusual factors, in regards to greenhouse practices, were the burned bark and the methyl bromide. Normally bark for such purposes does not contain wood fragments.

Moser (1949) has reported morels found growing at three places in a mine. The morels were found 100 to 200 meters underground. Wood was present in one place, one place was a latrine, and one place seemed to lack any kind of organic material. Moser found that there was much bacterial activity in all three places. No light was present in any of the places.

Production by Horticultural Methods

Some workers have reported obtaining sporocarps using horticultural methods. In 1901, Repin obtained morels from nine year old cultures that had been kept in flower pots in a cave. Repin also obtained sporocarps in dry leaves with added sodium carbonate and in a trench filled with apple mash (Brock, 1951). The Baron 'd'Yvoire's method required loose, moist, soil where Jerusalem artichokes were growing. He used whole fruiting bodies to innoculate beds in May and June. He watered during the summer with a potassium nitrate solution. In the fall he laid apple mash one cm deep and added a layer of dry leaves and twigs. In April and May the leaves and twigs were removed. He obtained a yield of 300 sporocarps in 100 square feet of bed (Brock, 1951; Baker and Matkin, 1959). Molliard obtained sporocarps in 20 days from a pot of earth to which he had added five kg of apple mash (Singer, 1961; Brock, 1951). Molliard also obtained morels from a bed that he made with old papers and apple mash. The bed was located under a row of cherry trees. Molliard used spores and mycelium from pure cultures as innoculum in his experiments. Constantine repeated the latter experiment and obtained 23 sporocarps from one square meter of bed (Cailleux, 1969; Singer, 1961). Cailleux (1969) reports that others have tried similar experiments, using apple mash and papers without success. Babee (1936) reported the appearance of Morchella sporocarps where he had mixed household garbage with earth and allowed it to decompose in a wooded area for one year.

Nutrition

Morchella may be grown on a wide variety of nutrient materials. Nitrogen may be supplied as proteins, amino acids, ammonium, nitrate, and

nitrite salts (Gilbert, 1960; Brock, 1951; Litchfield, 1967). Ammonium salts are usually a better nitrogen source than nitrate or nitrite salts (Brock, 1951). Not all proteins and amino acids are good nitrogen sources. Brock (1951) found that the amino acids l-cysteine-HCl, dl-aspartic acid, and l (+) asparagine are the best nitrogen sources. Gilbert (1960) found that proteins from peanut meal, coconut meal, soybean meal, and wheat bran, as well as albumin and peptone, are good nitrogen sources.

The minerals S, P, K, Na, Mg, Fe, are sufficient to maintain good growth for Morchella. The microelements Mn, Cu, Zn, Br, I, Cr, Mo, and B may be beneficial to Morchella (William and William-Engels, 1956). Robbins and Hervey (1959, 1965) have found that a wood extract stimulates growth of Morchella due to manganese and calcium in the wood ash. They also found that Morchella was able to utilize greater amounts of manganese than most fungi. It was found that Morchella is more resistant to acid conditions with the addition of manganese and calcium to the medium (Robbins and Hervey, 1965).

Morchella grows well on a wide variety of simple and complex carbohydrates. Starch, maltose, glucose, fructose, and sucrose are superior carbohydrates for nutrition of Morchella (William and William-Engels, 1956; Brock, 1951; Hurni, 1946). In addition, turanose, saccharose, xylose, lactose, the alcohols mannitol and glycerol, as well as food processing wastes are all suitable carbon-energy sources (Gilbert, 1960; Robbins and Hervey, 1965; Litchfield, 1967; Brock, 1951). Litchfield (1963) found that the ability to utilize lactose was variable, even among cultures obtained from the same origin.

Morchella has the best growth at a pH near neutral (Brock, 1951; Robbins and Hervey, 1959; William and William-Engels, 1956). Robbins and Hervey (1965) found that growth at a pH of 4.8 was equal to growth at a pH of 6.5, if sufficient manganese and calcium were present.

Morchella doesn't require an exogenous source of vitamins. William and William-Engels (1956) tested the response to pantothenic acid, biotin, thiamin, and pyridoxine. It was found that none of these vitamins affected the growth of Morchella. Hurni (1946) found thiamine to be slightly inhibitory. Hurni (1946) found that an apple extract stimulated growth. Hurni (1946) concluded that the stimulation was caused by malonic acid in the apple extract.

Gilbert (1960) reported that Morchella will grow at temperatures between 3 and 31° C, with the optimum temperature between 13 and 22° C. Falck (1920) reported that the growth rate increases rapidly as temperatures increase from 0 to 22° C, with the optimal growth rate at 24° C. He found growth to be severely inhibited at temperatures above 30° C. Hurni (1946) found that for periods of 15 days or less the optimum temperature was 25° C. Cultures grown for 20 to 30 days attained the greatest dry weight if they were grown at 16 to 18° C. Hurni (1946), William and William-Engels (1956), and Litchfield (1967) found that maximum growth was at 25° C.

William and William-Engels (1956) found that neither light nor dark affected growth of Morchella.

Composition

The cell wall of Morchella mycelium is mainly made up of protein, non-nitrogenous polysaccharides, chitin, and phosphate. Glucose,

mannose, and galactose are present in the cell wall (Herrara and Osario, 1974).

McKellar and Kohrman (1975) compared the amino acid composition of Morchella mycelium to Morchella sporocarps. They concluded that the amino acid composition varies mainly as a function of the nutritional conditions during growth. They did find differences in the amino acid composition of stipe and pileus tissue. The main differences are in the amounts of arginine and histidine.

The main minerals fround in Morchella sporocarps are potassium and phosphorus (Hawker, 1950; Cochrane, 1958).

MATERIALS AND METHODS

The study involved testing the response of Morchella to various nutritional variables. The nutritional variables that were tested included the concentration and source of nutrients. Three techniques were used to study the nutritional physiology of Morchella: agar cultures, submerged cultures, and cultures on peat pellets. The agar cultures were used for testing the response to different concentrations and types of nitrogen salts and sugars, variable concentrations of phosphates, potassium, and variable initial pH. Tests were performed on agar cultures to find the response to natural compounds present in potato tubers and Morchella sporocarps. The response to plant growth regulators was also tested using agar cultures. Submerged culture was used to test the response to vitamins. Cultures on peat were used to test the effect of fluctuating supplies of nitrogen, sugar, and complete nutrient solution.

The standard medium was made up of the salts and vitamins in Murashige and Skoog's medium (MS Medium)(Murashige and Skoog, 1962). The standard medium (see Table 3, Appendix A) includes a 0.05M phosphate buffer, 30 g sucrose/l., and the pH is 5.6. Ten g of agar per liter was used for agar cultures. Nitrogen is supplied as ammonium nitrate and potassium nitrate. When necessary pH was adjusted with 0.1 N HCl or 0.1 N NaOH. The pH was measured before sterilizing using a pH meter. Deionized water was used in this medium.

The basal medium for submerged cultured was the standard medium but with 0.2 M potassium nitrate, as sole nitrogen source. No vitamins were included in the basal medium. Glucose was substituted for sucrose

at 30 g/l. The medium was freshly prepared, using distilled water. Vitamins (NBC Biochemicals) were supplied in the following concentrations: biotin, 2 µg/l; calcium pantothenate, 400 µg/l; folic acid, 2 µg/l; inositol, 2000 µg/l; niacin, 400 µg/l; para amino benzoic acid, 200 µg/l; pyridoxine HCl, 400 µg/l; riboflavin, 200 µg/l; thiamine HCL, 400 µg/l.

Sterilization of all types of media was accomplished by autoclaving for 15 minutes at 15 psi and 120^{o} C.

Medium for agar cultures was prepared by mixing all components of the medium. The medium was then heated approximately five minutes in an autoclave, to dissolve the agar. The medium was then poured into the culture vessels which were sealed with aluminum foil, and the medium was sterilized. Culture vessels were 125 ml Erlenmeyer flasks with 50 ml of medium added to each flask. There are two exceptions to this method of making medium. Medium for testing pH was prepared by autoclaving buffer separately from the medium. The buffer was added aseptically after autoclaving to prevent breakdown of agar or sugar. A solution of each vitamin was sterilized by filtration and appropriate amounts of the medium and the desired vitamin were then combined.

Potato juice was obtained by freezing fresh potato tubers. After thawing, the juice was squeezed out through cheesecloth. A homogenate of ground Morchella sporocarps and water was prepared using a Waring blender. Both the potato juice and the sporocarp homogenate were added to the medium before autoclaving. The response to ethylene was tested aseptically by adding ethylene to 21 day old cultures. Other plant growth regulators were added to the medium before autoclaving.

Agar cultures were grown 21 days. The cultures were kept in an incubator in the dark at 25° C.

Peat cultures utilized peat pellets (Jiffy Seven, Jiffy Pot Ltd., Grorud, Norway). A peat pellet is a cylinder of peat approximately 4.5 cm in diameter and 4 cm in height, and is held together by fiberglass cloth. The peat pellets served as a mechanical support for the growing mycelium. The peat pellets were placed in 400 or 600 ml beakers, to which 50 ml of liquid medium had been added. The purpose of the peat cultures was to test the response to fluctuating nutrient supply. This was achieved by transferring the peat pellets upon which mycelium was growing to a beaker containing fresh medium. This was done twice a week. The composition of the medium was changed slightly at each transfer. Three different variables were tested in this manner: concentration of the complete nutrient medium, nitrogen concentration, and sugar concentration. Cultures receiving variable levels of the nutrient solution started at 0.1X the normal concentration of the standard medium. The concentration was gradually increased until it was 2X at two and one-half weeks. From two and one-half weeks to the fifth week the concentration was reduced until the cultures were transferred to deionized water for the final transfer, at the beginning of the fifth week. Experiments with variable levels of nitrogen (with ammonium nitrate as the nitrogen source) and with variable levels of sugar were done in a similar manner. Cultures for the variable nitrogen test started on 0.05 M ammonium nitrate plus the normal components of the medium. At two and one-half weeks the cultures received 0.02 M ammonium nitrate and on the final

transfer, at the fifth week cultures were transferred to nitrogen-free medium. Cultures receiving variable levels of sucrose started with 5 g/l, were gradually transferred to medium containing 100 g/l at two and one-half weeks, and from the fifth week on they had no sugar in the medium. The controls in these three tests received the same total amount of the varied nutrients as those receiving a variable medium but in equal amounts at each transfer. Except for the tested components of the medium, the standard medium was used. The medium for peat cultures was buffered at pH 6.8 with a phosphate buffer, at a concentration of 0.01 M phosphate.

In each test (peat cultures) a group of cultures was incubated eight weeks at 25° C and another was kept at 17° C.

Submerged culture was used for testing the response to vitamins. The culture vessels were 125 ml Erlenmeyer flasks with 40 ml of medium in each flask. All glassware used in preparation and culturing was washed with hot 1.0 N HCl and rinsed with distilled water. Glass distilled water was used in preparing the medium. Cultures were allowed to grow five days at room temperature, with constant shaking on a reciprocal shaker. The shaker was set at 100 r.p.m.

The inoculum originated as a piece of stipe tissue from a wild M. esculenta sporocarp. Inoculum was maintained on rye and on agar plates. Rye cultures served as a bank of inoculum and have been found to remain viable for two years or more, when refrigerated at approximately 5° C. Inoculum to be used for starting cultures on test media was maintained on petri plates of potato dextrose agar, or the basal medium for submerged culture. Difco special (purified) agar was used to make plates with the

basal, submerged culture medium. Twenty four grams of dehydrated potato dextrose agar was used to make one liter of medium. Approximately 30 ml of medium was used in each plate. The petri plates and the medium were first sterilized. The medium was then poured, just before the agar began to solidify. Cultures on potato dextrose agar served as inoculum for agar and peat media. The inoculum on basal submerged culture medium was used to inoculate liquid medium for submerged cultures. Inoculum from the second subculture served to inoculate the medium for vitamin tests in submerged culture. A piece of medium approximately 0.5 cm square, with the mycelium growing on it, was cut from the plates, this was then used to inoculate medium.

Inoculating and transferring was done in a forced air hood. Tools used in inoculating and transferring were dipped in 70% ethanol and flamed each time they were used. Cultures were evaluated according to their final dry weight and whether any morphological changes were induced.

The dry weight was obtained after drying 8 to 10 hours at about 100° C. Mycelial pads could be removed in one piece from agar and submerged cultures (Figure 3). To remove mycelium from agar cultures it was first necessary to melt the agar by heating the flasks on a hot plate. The mycelial pads were then rinsed in hot water and dried. Mycelium in submerged cultures grew together in aggregates that could easily be removed with a spatula and then dried. After drying, mycelium was weighed using an analytical balance.

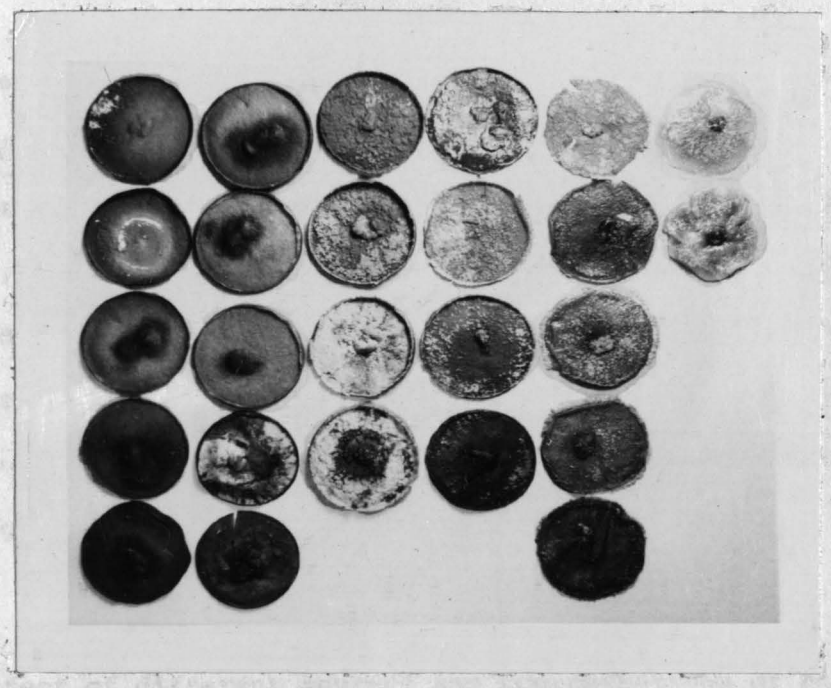

Figure 3. Typical mycelial pads before drying and weighing. Five replicates of each treatment were weighed except in cases where contamination occurred. 1/4 X.

Mycelia from peat cultures were evaluated according to their growth habit and appearance. Photographs of peat cultures were made in order to have a record of their appearance.

The criteria for evaluating sclerotia production were the number of days before sclerotia began to appear and the surface area covered by sclerotia after 21 days. In some cases it was necessary to estimate the surface area covered by sclerotia by photographing the mycelial pad. The photographic negative was then placed in a photographic enlarger and projected onto graph paper.

RESULTS

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

Analysis of variance indicates that differences due to treatment effects exist, when tested at the 0.01 level for all experiments that were evaluated quantitatively (Tables 4 through 13, Appendix B). This includes tests of nitrogen source and level, variable concentration of potassium, phosphorous, and sucrose, variable initial pH, kind of carbohydrate, and growth with vitamins.

Nitrogen

The effect of different sources and concentrations of nitrogen was tested. The different nitrogen sources were supplied in the following concentrations: 0.001, 0.005, 0.01, 0.05, and 0.1 M. The results are summarized in Figure 4. The highest dry weight, 666 mg, was obtained using ammonium nitrate at a concentration of 0.01 M. No cultures grew on medium with 0.1 M ammonium nitrate. When the nitrogen source was ammonium tartrate, at a concentration of 0.01 M the mean dry weight was 454 mg. When the medium contained 0.01 M ammonium chloride the dry weight was 297 mg. The highest dry weight, using sodium nitrate, at a concentration of 0.05 M, was 348 mg. Tukey's omega test at the 0.05 level indicates ammonium nitrate is better than any other tested nitrogen source when they are present at 0.01 M (Tables 14 and 15, Appendix C). At a concentration of 0.1 M Tukey's omega test indicates that sodium nitrate is better than any other nitrogen source. The source x level

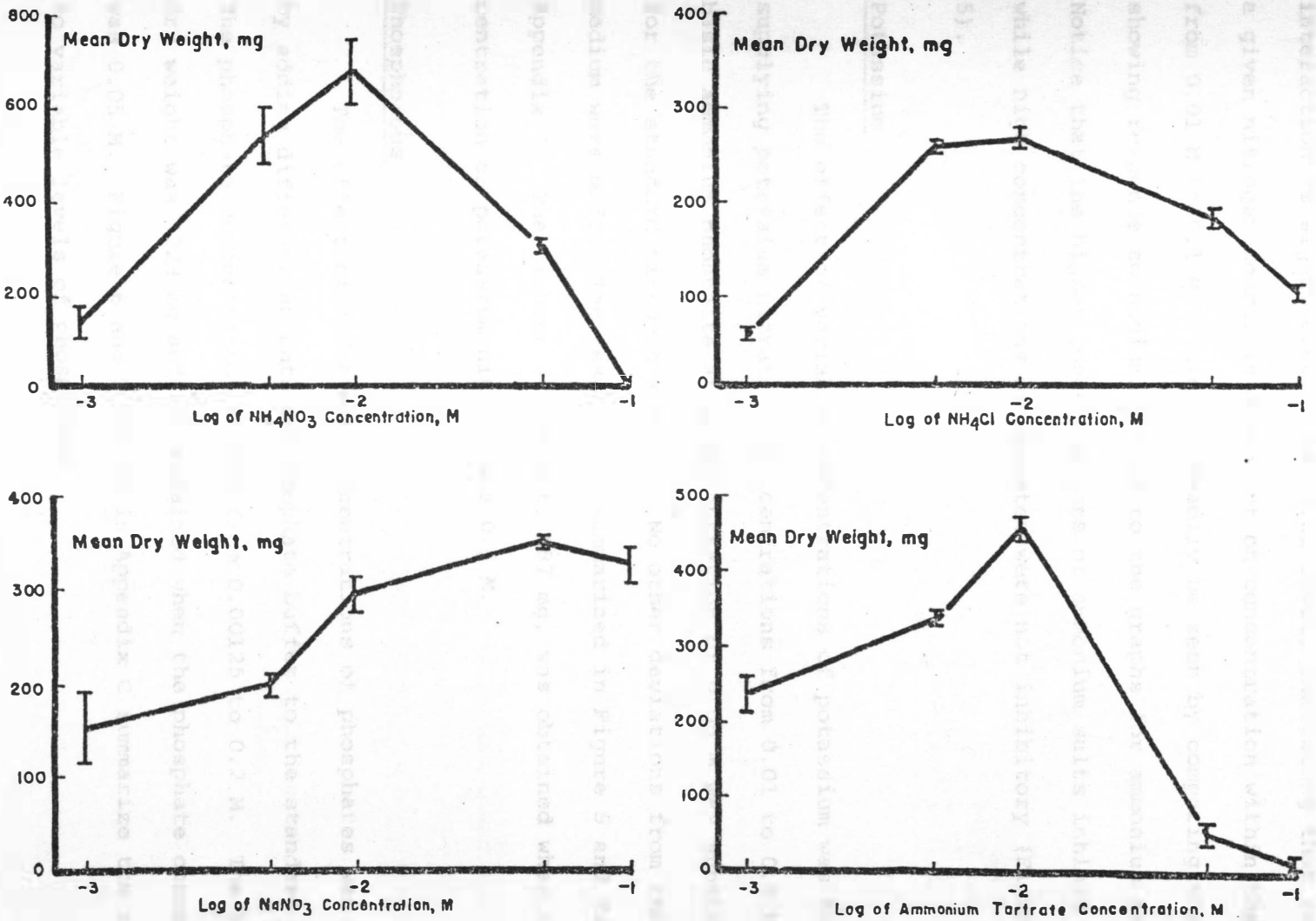

Figure 4. Growth of Morchella mycelium with four nitrogen sources supplied at five concentrations. Vertical bars in all graphs indicate standard deviations.

interaction is significant at the 0.01 level, indicating that response to a given nitrogen source is dependent on concentration within the range from 0.01 M to 0.1 M. This can readily be seen by comparing the graph showing response to sodium nitrate to the graphs for ammonium salts. Notice that the higher concentrations of ammonium salts inhibit growth while high concentrations of nitrates were not inhibitory (Figures 4 and 5).

Potassium

The effect of variable concentrations of potassium was tested by supplying potassium nitrate in concentrations from 0.01 to 0.5 M. Monobasic ammonium phosphate at a concentration of 0.05 M was substituted for the standard nitrogen sources. No other deviations from the standard medium were made. The results are summarized in Figure 5 and Table 16 in Appendix C. The highest dry weight, 687 mg, was obtained when the concentration of potassium nitrate was 0.5 M.

Phosphorous

The effect of different concentrations of phosphates was obtained by adding different amounts of phosphate buffer to the standard medium. The phosphate concentration ranged from 0.00125 to 0.2 M. The highest dry weight was 1223 mg and was obtained when the phosphate concentration was 0.05 M. Figure 6 and Table 17 in Appendix C summarize the response to variable levels of phosphorous.

pH

The response to media having different pH was tested with ammonium tartrate at 0.01 M concentration as the nitrogen source. Varying amounts

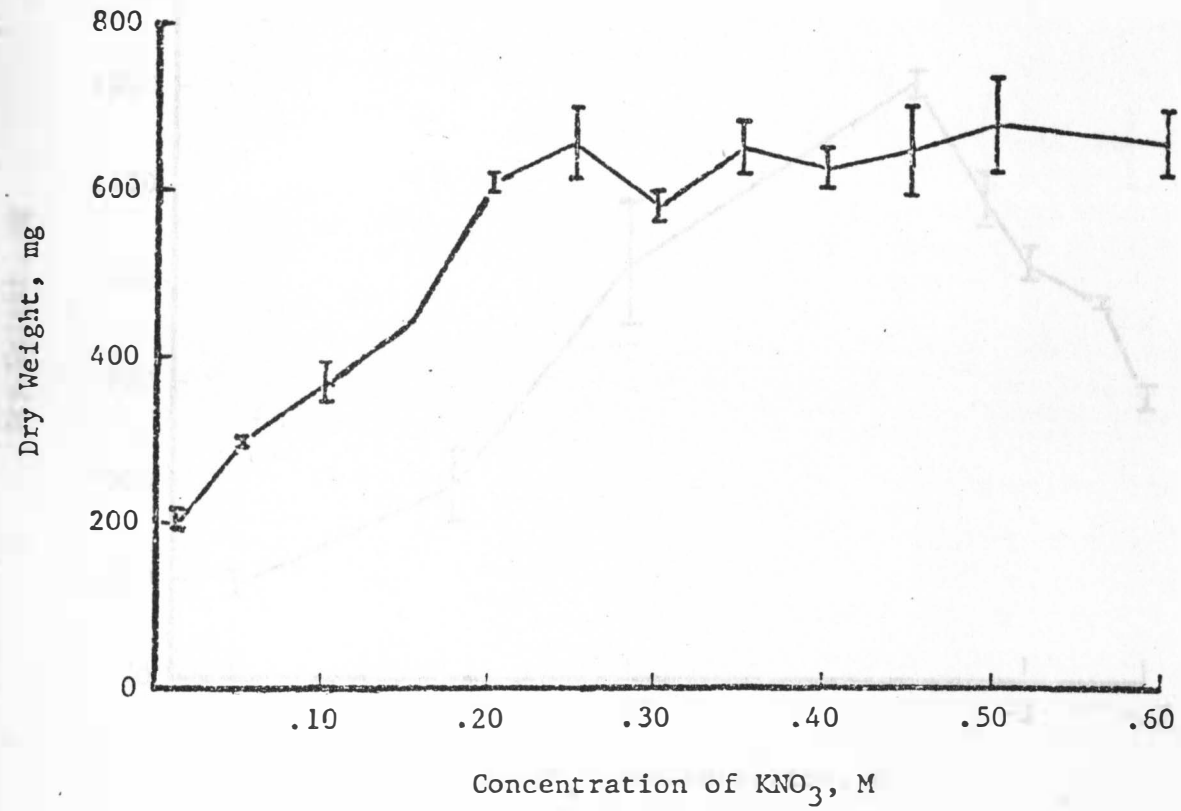

Figure 5. Relationship between growth of Morchella and the concentration of potassium nitrate in the medium.

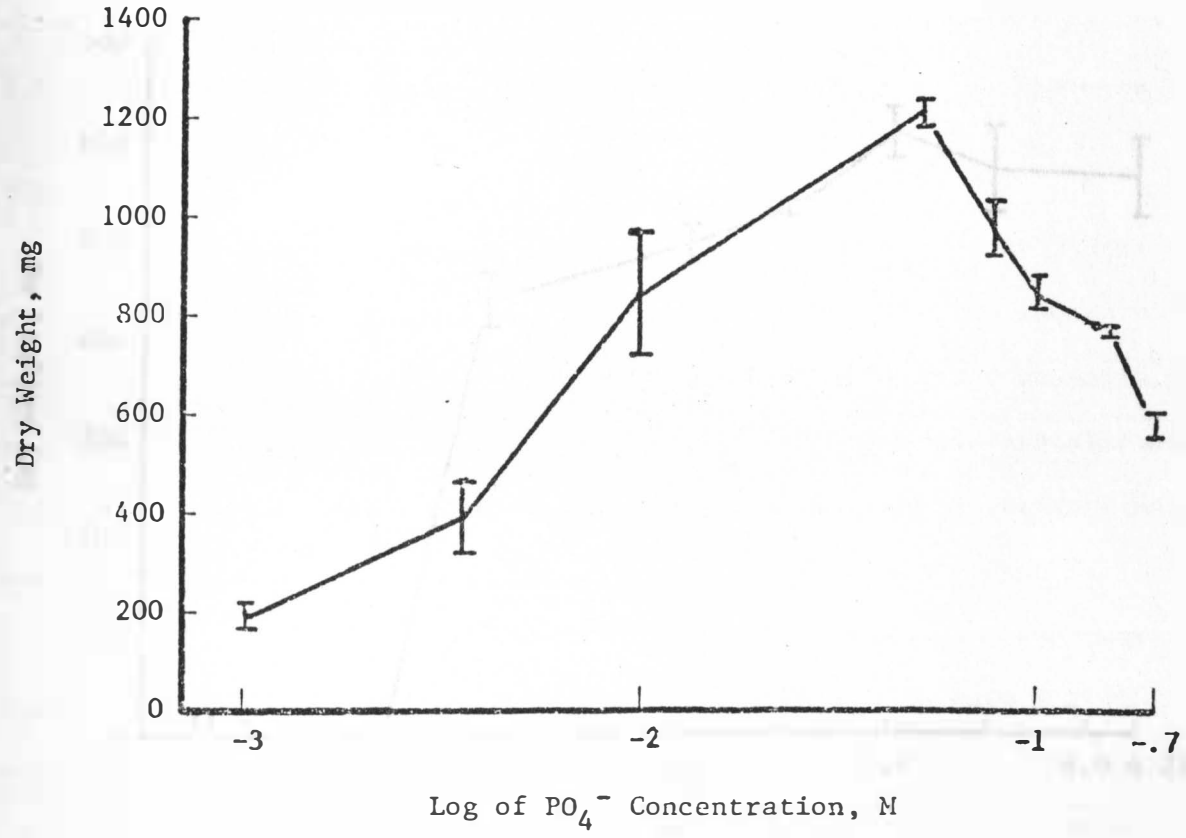

Figure 6. Growth of Morchella with different concentrations of phoshpate ion in the media.

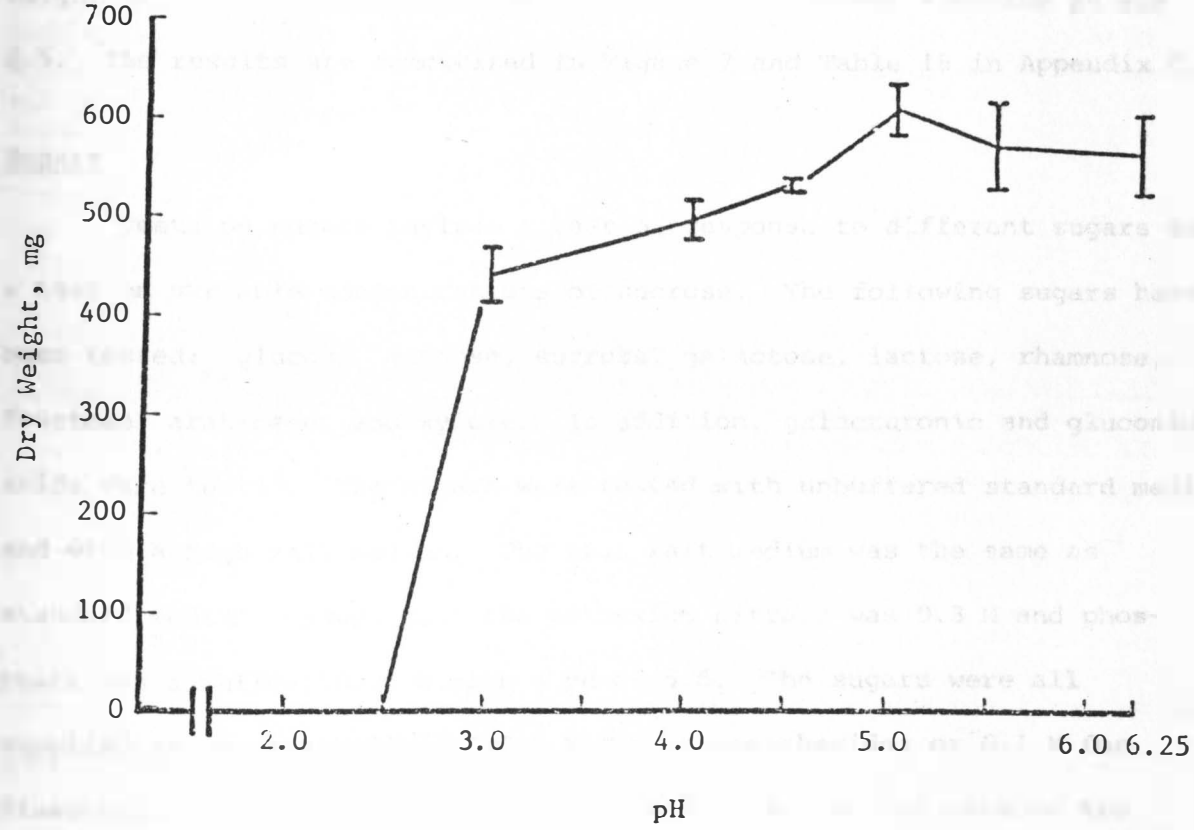

Figure 7. Growth of Morchella on media having variable initial pH.

of citric acid was added to alter the pH. The concentration of citric acid varied from 0 to 0.214 M. A phosphate buffer having a pH of 6.25 and a phosphate concentration of 0.05 M was used at all pH ranges. The greatest dry weight was found when the initial pH was 5.0. The dry weight at a pH of 5.0 was 612 mg. There was no growth when the pH was 2.5. The results are summarized in Figure 7 and Table 18 in Appendix C.

Sugars

Tests on sugars include a test on response to different sugars and a test on variable concentrations of sucrose. The following sugars have been tested: glucose, mannose, sucrose, galactose, lactose, rhamnose, fructose, arabinose, and xylose. In addition, galacturonic and gluconic acids were tested. The sugars were tested with unbuffered standard media and with a high salt medium. The high salt medium was the same as standard medium, except that the potassium nitrate was 0.3 M and phosphate (as a buffer) 0.05 M with a pH of 5.6. The sugars were all supplied at concentrations of 0.2 M for monosaccharides or 0.1 M for disaccharides. On unbuffered standard media glucose and mannose are better than all other sugars, while sucrose is better than the remaining sugars except galactose, as indicated by Tukey's omega test at the 0.05 level (Table 19, Appendix C). On media with increased NPK Tukey's omega test at the 0.05 level indicates that mannose is better than all other sugars except sucrose (Table 20, Appendix C). Sucrose, glucose and fructose are found to be better than the remaining sugars. The results of the tests on variable carbohydrate sources are summarized in Table 1.

Table 1. Growth with Different Carbohydrates and with Two Nutrient Salt Solutions.

Carbohydrate	Standard medium (unbuffered) Mean, mg	S.D.	Increased NPK 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
No Carbohydrate	---	---	---	---

* All sugars are (D)+ except ribose and arabinose which are (D)-. From United States Biochemical Corporation, Cleveland, Ohio.

** Dry weight.

The response to the concentration of sugar was tested using sucrose as the carbon-energy source. The sucrose concentration ranged from 5 to 200 g/l. The greatest dry weight, 527 mg, was obtained when the medium contained 100 g/l of sucrose. Figure 8 and Table 21 in Appendix C show the results of this test.

Response to Fluctuating Nutrient Levels

Cultures on peat pellets were evaluated according to morphology only. There was no difference in the form of growth, regardless of the treatment. All peat cultures developed a large mass of sclerotia at the top, with mycelium spreading out across the surface of the medium as seen in Figure 9.

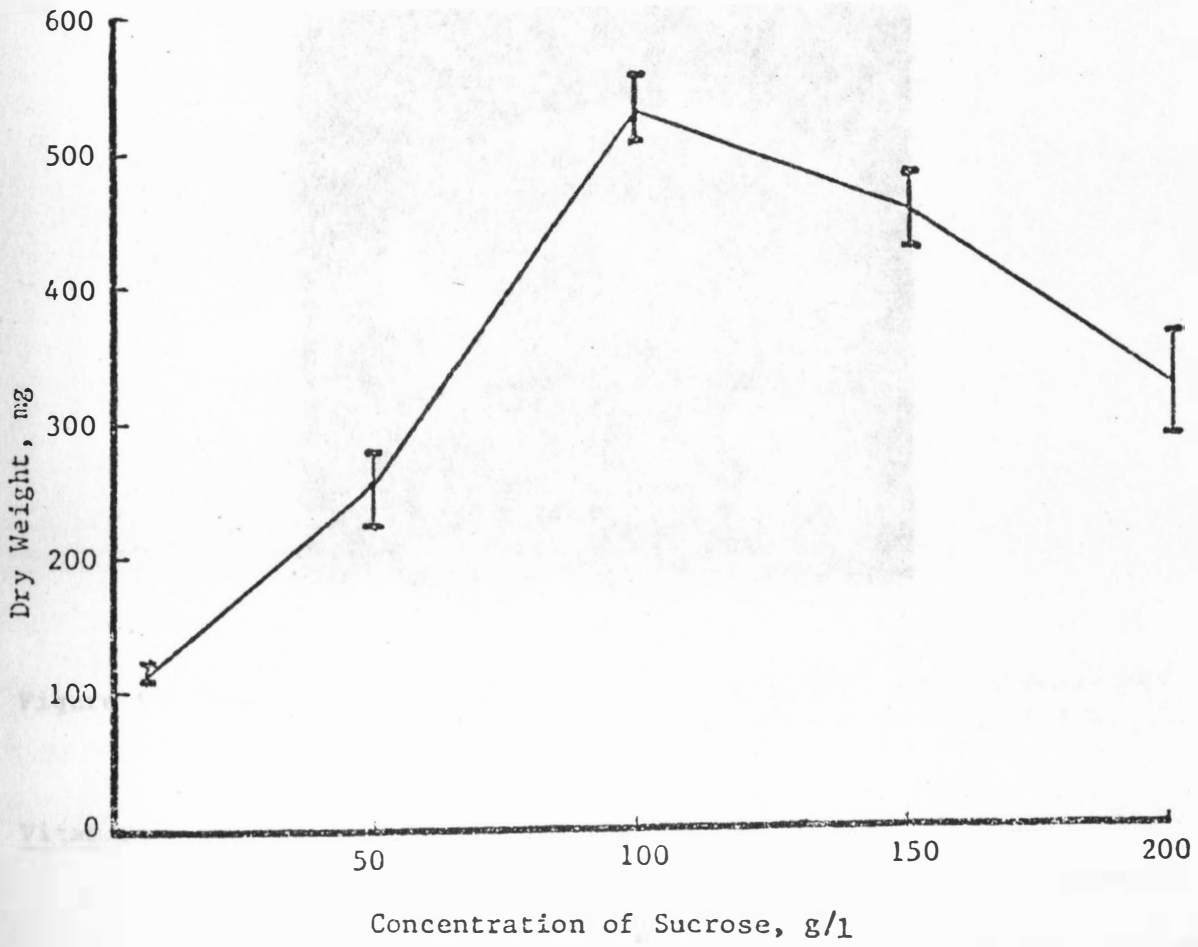

Figure 8. Relationship between growth of Morchella and the concentration of sucrose in the medium.

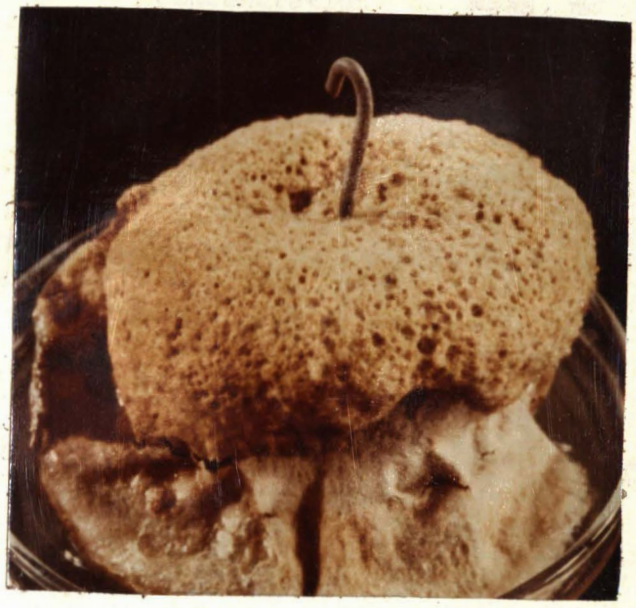

Figure 9. Typical culture growing on a peat pellet after 4 weeks of growth. Note large amount of sclerotia on top. 1/4 X.

Vitamins

There were 11 treatments in the vitamin study. Nine treatments had a single vitamin, or inositol, in the medium. One treatment included all eight vitamins and inositol. No vitamins were included in the control medium. 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 growth among replicates within a treatment, as is indicated by the standard deviations. The control cultures had relatively uniform amounts of growth. Dunnet's test at the 0.05 level, indicates no differences due to treatment effects between vitamin treatments and the control (Table 24, Appendix C).

Table 2. Growth with Different Vitamins Added to the Media.

Vitamin Added	Concentration	Mean, mg	S.D.
Nicotinic	400	0.14	0.31
Thiamine	400	0.52	0.75
Inositol	2000	5.56	5.19
Biotin	2	29.38	17.66
P-aba	200	40.14	15.25
Pyridoxine HCl	400	49.28	14.84
Ca Pantothenate	400	51.14	9.74
Riboflavine	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

Organic Compounds

The effect of some undefined organic compounds was tested by adding potato juice and a homogenate of fresh Morchella sporocarp tissue to the medium. The potato juice was added in concentrations from 0 to 75% (v/v). One liter of potato juice was obtained from 2 kg of tubers. On a medium containing 25% (v/v) potato juice the dry weight was 775 mg, while the dry weight with no potato juice was 93 mg. Results are summarized in Table 22 in Appendix C. When adding the homogenate of Morchella sporocarp tissue it was hoped that a hormone-like substance, might be present in the tissue. The concentration of sporocarp tissue in the medium ranged from 0 to 10% (fresh w/v). The highest dry weight, 425 mg, was found on medium containing 10% sporocarp tissue. The dry weight on medium with no sporocarp tissue was 96 mg. Results are summarized in Table 23 in Appendix C. No unusual growth forms were noted when sporocarp tissue was added to the medium except that the tendency to

produce sclerotia seemed to be proportional to the concentration of sporocarp tissue.

Plant Growth Regulators

The response to the vascular plant growth regulators (IAA, kinetin, giberellic acid, and ethylene) was tested. No response was noted when IAA, kinetin, and giberellic acid were tested at 10 ppm. When the concentration was 100 ppm, IAA had a slightly inhibitory effect, while kinetin and giberellic had no effect. When ethylene was added to 21 day old cultures, at concentrations of 0.1 and 1 ppm, no response was observed.

Nutritional Factors Influencing Sclerotia Formation on Agar Media

Morchella mycelium may produce sclerotia under certain conditions (Figure 10). Sclerotia are occasionally produced on the standard medium. Some factors that may influence sclerotia formation are nitrogen source and concentration, concentrations of phosphate and carbohydrate, and pH.

The source of nitrogen has a strong influence on sclerotia formation. When ammonium compounds are included in the medium, sclerotia formation and growth is inhibited. Sclerotia are rarely produced on the standard medium, which includes ammonium nitrate at a concentration of 0.02 M. The tendency to produce sclerotia is inversely proportional to the concentration of ammonium salts, over the range of concentrations tested. When medium containing ammonium salts is buffered, there is a greater tendency to produce sclerotia. More sclerotia were produced when calcium carbonate was the buffer than when a phosphate buffer was used.

The source of nitrogen is apparently less important when cultures are grown on peat. Cultures on peat produced a large amount of sclerotia when the ammonium nitrate concentration was 0.02 M. Cultures on agar would have little tendency to produce sclerotia if ammonium salts were present at this concentration.

Sclerotia are produced in large numbers when potassium nitrate is the sole nitrogen source. When potassium nitrate is supplied at concentrations of 0.03 to 0.06 M, 87% to 95% of the surface area was covered by sclerotia. However, the cultures had little tendency to produce sclerotia unless potassium nitrate was the sole nitrogen source. The number of sclerotia produced increases with an increase in potassium nitrate concentrations from 0.02 M to 0.03 M. At higher concentrations, within the range tested, potassium nitrate concentration had little effect on sclerotia production.

Sclerotia were rarely found where the final pH of the medium was less than five. The final pH of medium upon which largest numbers of sclerotia were found was near neutral. In one test the initial pH of the standard medium was buffered in a pH range from 5 to 8 using phosphate buffers. The final pH ranged from 3 to 7. No sclerotia were produced, regardless of pH. The final pH may reflect utilization of different nitrogen sources and not be directly related to sclerotia formation.

When phosphate buffers are added to media that would normally enable the production of large numbers of sclerotia, the numbers of sclerotia are reduced. No sclerotia were produced when phosphate buffer at pH 5.6 and a concentration of 0.05 M was added to the five best

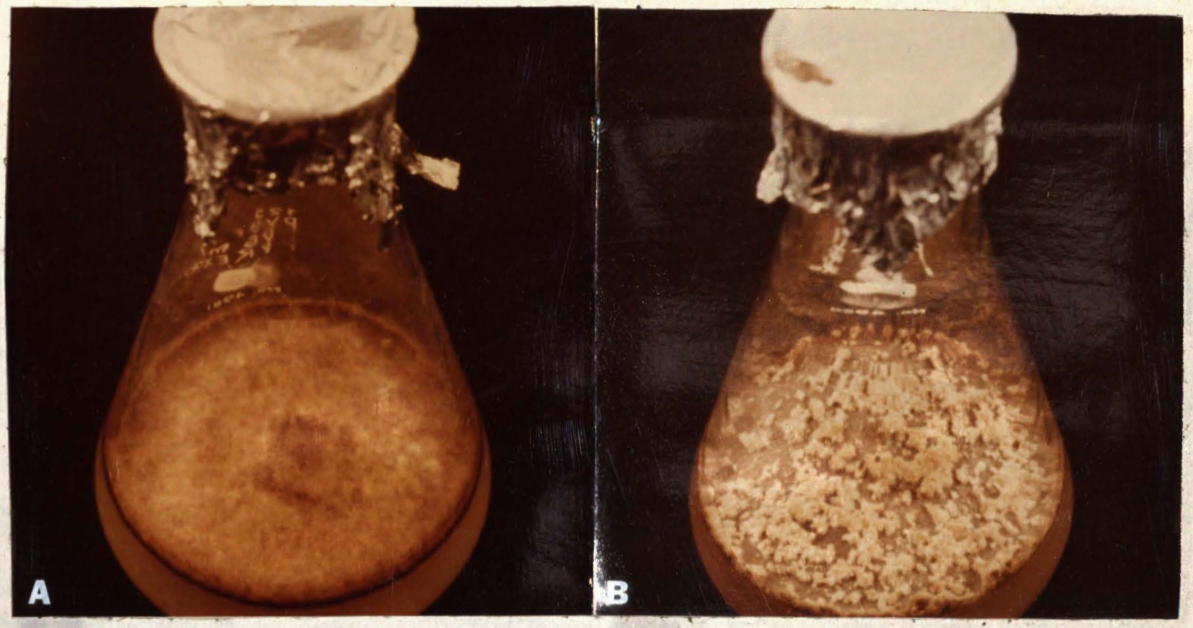

Figure 10. Mycelial pads that formed on agar. (A) No sclerotia have formed. (B) Culture that has formed large numbers of sclerotia. 1/4 X.

"sclerotia-producing" media. At a concentration of 0.01 M phosphate, some sclerotia were produced.

Analysis of Tissues by Microscope

Mycelium and sclerotia from several cultures were analyzed microscopically (Figure 11). Conventional paraffin techniques of dehydrating, embedding, and staining were used. Sections were triple-stained with safranin, crystal violet, and fast green. The appearance of paraffin sections of mycelium is essentially unaffected by nutritional variables. Only vegetative hyphae have been observed by microscopic examination. Sclerotia are found to be made up of pseudoparenchymatous tissue.

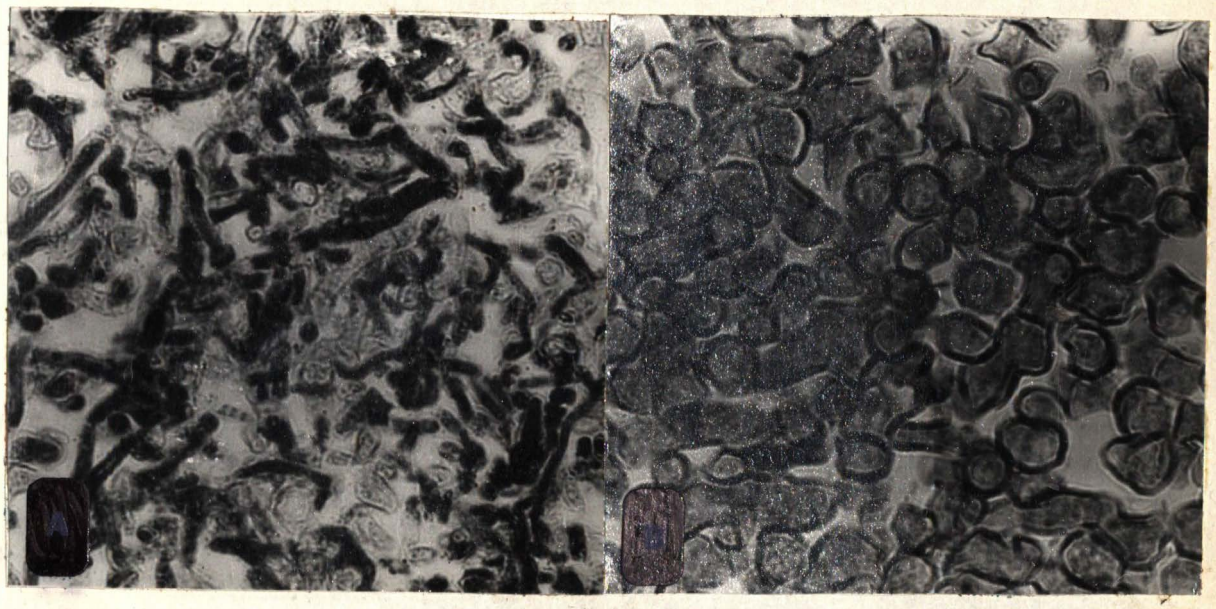

Figure 11. Paraffin sections of (A) mycelial pad and (B) sclerotia. Note isodiametric cells of B and elongated hypheal cells in A. 100 X.

DISCUSSION

Nitrogen Source and Concentration

All nitrogen sources tested are capable of supporting growth by Morchella. The highest yields were achieved with ammonium nitrate as the nitrogen source.

Ammonium salts give rise to maximum growth at relatively low concentrations, but higher concentrations are likely to be inhibitory. Nitrates can support comparable growth, but at much higher concentrations. There is apparently no toxicity by the nitrate ion, and it has been present in the media at concentrations as high as 0.6 M with no apparent inhibition. Cultures growing on media with ammonium salts present are less likely to produce sclerotia than if potassium nitrate serves as the sole nitrogen source.

Most fungi can utilize either ammonium or nitrate as nitrogen sources (Ainsworth, 1965). When both ammonium and nitrate are present the ammonium is usually used first. The uptake of ammonium ion is believed to occur by passive diffusion in many fungi. Uptake of nitrate is suppressed by the presence of ammonium, in many fungi, by blocking the reduction of nitrate to nitrite (Morton and Macmillan, 1954). Many fungi are unable to completely utilize ammonium salts on unbuffered media. The ammonium ion is liable to be taken up much faster than its associated anion, resulting in inhibitory acid conditions. Morton and Macmillan (1954) have reported this phenomenon for Scopularis brevialis. They found that the organism could not completely utilize ammonium sulfate unless buffers were added to the media. Brock (1951) found this pH change for

Morchella, as did Robbins and Hervey (1959). In the present study it was found that cultures on unbuffered media with ammonium salts present and an initial pH of 5.6 grew poorly and had a final pH of 3 or less, after 21 days of incubation.

Brock (1951) found ammonium salts to be better than nitrate salts (at 250 mg N/l), but several amino acids and sodium nitrite were better than or equal to any of the ammonium salts he tested. This generally agrees with the data obtained in this study, except that at a concentration approximately equal to 250 mg N/l the rank (in descending order) would be ammonium tartrate, sodium nitrate, ammonium nitrate, ammonium chloride. Brock's study would give the following ranking: ammonium tartrate = ammonium nitrate, ammonium chloride, sodium nitrate. Possible reasons for the differences between the two studies include differences in the basal medium, differences in incubation time (six days for Brock's experiments), genetic differences between cultures, Brock's study utilized liquid cultures, and Brock's media had an initial pH of 6.5 to 7.

Carbon Source

In this study glucose, mannose, and sucrose were found to be the three best sugars, with two different media. The relative amounts of growth were somewhat different with the two media. Some sugars supported somewhat different amounts of growth on unbuffered, standard medium vs. the medium having increased NPK levels. Sucrose, fructose, arabinose, and xylose gave rise to much more growth on the medium with increased NPK. The increased level of phosphorous may have enhanced the ability to utilize these sugars. Rhamnose and gluconic acid gave better results with the unbuffered standard medium.

There is a wide range in the amount of growth among the different carbon sources, but all the sugars are suitable with at least one of the two media tested. Galacturonic and gluconic acids gave poor results on either media.

Results for the sugars tested is in general agreement with results obtained by William and William-Engels (1956), Litchfield (1967), and Brock (1951). William and William-Engels (1956) found the sugars, sacharose, glucose, and maltose, to be the best. Brock found sucrose, fructose, turanose, glucose, and maltose best. Litchfield notes a number of sugars which are satisfactory, including glucose, lactose, maltose, sucrose, and xylose. Differences between the findings of different researchers may reflect differences in enzyme activity of various strains. Litchfield (1963) found differences in the ability to utilize lactose, even if the cultures had arisen from the same sporocarp. Gilbert (1960) found differences between cultures whose mycelium arose from the same sporocarp for a number of characteristics including growth rate and appearance of the mycelium.

Growth of the mycelium was found to increase as the sucrose concentration increased, up to 100 g/l. Growth was found to be reduced at higher sucrose concentrations. The reduced growth at higher concentrations is probably an effect of osmotic pressure (Hawker, 1950). Increased availability of sucrose probably has a greater influence than osmotic pressure, up to 100 g/l.

Potassium

Growth was found to increase steadily with increased potassium nitrate concentrations to 0.25 M. Inhibition was not observed with concentrations as high as 0.6 M.

Since monobasic ammonium phosphate was present at a concentration of 0.05 M, the nitrate ion may not have been significant as a nitrogen source. The high relative concentration of nitrate ion to ammonium ion could possibly increase the likelihood of nitrate uptake, however.

Phosphorous

Increasing the phosphate concentration up to 0.05 M was associated with steadily increased growth. Growth dropped off at higher levels of phosphate. Since the phosphate was supplied as dibasic sodium phosphate and monobasic potassium phosphate, the benefit to growth was probably closely associated with the buffering ability of the phosphate salts. With the phosphate concentration at 0.00125 M the final pH was 3, at 0.01 M the final pH was 3.5, and at 0.05 M the final pH was 5.

pH

In this study good growth was found at a wide range of pH, from 3 to 6.25. The greatest growth was found when the pH was 5.0. Most researchers have reported the best growth at a pH near neutral to slightly alkaline (William and William-Engels, 1956; Brock, 1951; Robbins and Hervey, 1959). Brock (1951) reported the highest growth to occur when the initial pH was 6.93. Litchfield, Overbeck and Davidson (1963) reported the best growth when the initial pH was 6.5. The results of

this study are somewhat in conflict with the results obtained by other people. Possible reasons for the different results include genetic differences, nitrogen source, duration of incubation, salts included in the media, and liquid vs. agar culturing effects. Differences due to the nitrogen source and to the salts included in the media are espeically plausible. As has been mentioned before, ammonium salts tend to result in acid conditions, while the pH is likely to increase when the sole nitrogen source is nitrate. Robbins and Hervey (1965) found that the growth response to pH is much different if microelements, especially manganese and calcium are supplied in the medium. It was found that certain amino acids could be effectively utilized at a pH of 4.8 only in the presence of manganese and calcium. In addition, Robbins and Hervey (1965) found that the growth at pH 4.8 was equal to the growth at pH 6.5, if large amounts of manganese and calcium were used in the medium. Brock's medium did not include manganese or calcium.

Related to pH effects in pure cultures are reports of the soil pH where Morchella sporocarps have been found. A number of researchers have reported that sporocarps were found on soil with near neutral pH (Grainger, 1946; Sanderson, 1969) while Delmas and Bunel (1974) reported sporocarps on soil with pH from 5.5 to 8. It should be noted that these soil pH readings may be an indication of such factors as composition of soil, soil fertility, organic matter content, drainage, and climate; as well as an indication of a range of pH that permits reproductive growth. The pH tolerance may also indicate differences among strains or species.

Nutrient Availability

The availability of nutrients is a controlling factor in the change from vegetative to reproductive growth for many fungi. Sexual and asexual reproduction often occurs when a vigorous mycelium exhausts its nutrient supply (Cochrane, 1958; Morton, 1967). Fruiting of fungi is sometimes stimulated by a transfer from rich to dilute media (Hawker, 1957). Not all fungi sporulate when nutrients become limiting. Cytaria gunii fruits in response to the initiation of new spring growth by its host, the beech tree (White, 1954). Penicillium and Aspergillis are stimulated to sporulate in the presence of abundant assimilable carbohydrates (Ainsworth, 1966).

In this study no morphological changes were observed to occur due to changes in nutrient availability. Mycelium cultured on peat was exposed to limiting nutrients followed by optimal nutrients and then by conditions of limited or no nutrients. No differences were observed in the growth habits or appearance of cultures regardless of the nutrient supply. The above phenomena may apply to the induction of fruiting of Morchella, but the results of this study indicate that availability of nutrients is not an overriding factor in the induction of reproductive growth by Morchella.

Other Factors

Many fungi are able to synthesize all the required vitamins (Ainsworth, 1966; Hawker, 1957). In some cases vitamins are synthesized at adequate levels for vegetative, but not reproductive growth (Cochrane, 1958; Ainsworth, 1966; Hawker, 1957). Morchella appear to show no

requirement for exogenous vitamin supplies in this study. Thiamine, inositol, and nicotinic acid have been found to inhibit growth, at the concentrations used in this study. A number of vitamins tested, especially calcium pantothenate and pyridoxine, allowed for greater amounts of growth than the control medium with no vitamins, but no differences between vitamin treatments and the control could be judged significant by statistical analysis. The results of this test are supported by findings of other studies. Hurni (1946) found an inhibition with the addition of thiamine. William and William-Engels (1956) reported that vitamins thiamine, pyridoxine, pantothenic acid, and biotin did not influence growth.

Some fungi may be stimulated to sporulate by diffusible substances that may originate from plants, bacteria, other fungi, or like fungi. This may be due to effects on the nutrient media (pH, reduction of nutrient availability) or to the presence of organic, growth regulating compounds (Cochrane, 1958; Hawker, 1957; Asthana and Hawker, 1936; Leonian and Lilly, 1937). The effect may be due to an inhibition of vegetative growth or the induction of reproductive growth. The only growth regulator of this type that has actually been isolated is coprogen. Coprogen is produced by certain fungi and actinomycetes and stimulates reproductive growth in Pilobus (Cochrane, 1958). In this study Morchella was found to respond to factors present in potato tubers by increased growth. Factors present in a homogenate of Morchella sporocarps were found to enhance the tendency to produce sclerotia and increase the amount of growth. The responses observed may be due to common

nutrients supplied in the potato or sporocarp homogenate. Apparently the sporocarp homogenate contains a substance that promotes sclerotia formation.

If a growth factor is needed to induce or permit reproductive growth, it is probably a substance found commonly in many diverse habitats. Such a substance may be available to Morchella in larger quantities at times when plants are weakened by environmental stress. Mushroom hunters have known for many years that large numbers of morels are likely to be found on burned-over land. In more recent years morels have been found in large numbers near elm trees that are infected by Dutch Elm disease. Roze reported finding Morchella esculenta parasitizing rhizomes of Jerusalem artichoke (Singer, 1960). Morchella may need to grow as a parasitic form or a mycorrhizal form to enter a reproductive phase. Singer (1961) has suggested that Morchella may be a facultative parasite or a facultative mycorrhizae. If Morchella does need certain growth factors it may get these substances by growing as a parasitic or mycorrhizal form. This could explain the unusual numbers of morels found following situations where large numbers of plants have been injured. Weakened plants might be expected to leak contents of the cytoplasm through their membranes, thus enhancing a mycorrhizal type of growth, or the weakened plants might be parasitized by Morchella. Saprophytic fungi often will grow parasitically when they come in contact with a sufficiently weakened plant.

Sclerotia

The development of sclerotia under certain nutritonal conditions has been the only morphogenetic change observed in this study.

In this study the primary factors that were observed to affect sclerotia production are the nitrogen source and concentration, and the concentration of phosphate. Sanderson (1969) observed different tendencies to produce sclerotia among three different mycelial strains. Sanderson (1969) also found a relationship between the sucrose concentration and sclerotia production. In the present study sclerotia were consistently formed when the sole nitrogen source was potassium nitrate, and occasionally small numbers were produced when nitrogen was supplied as ammonium salts. Production of sclerotia by some other fungi is influenced by the source of nitrogen. Sclerotinia rolfsii requires an organic form of nitrogen to form sclerotia (Hawker, 1957). Botrytis cinerea will not produce sclerotia when supplied with potassium nitrate, peptone, or asparagine (Hawker, 1957). Sclerotinia sclerotiorum will produce sclerotia if supplied with potassium nitrate or an organic nitrogen source, but produces few sclerotia when the nitrogen source is an ammonium salt (Hawker, 1957). Morton (1967) has described morphogenesis of Neuspora crassa depending on whether nitrogen was supplied as ammonium or nitrate. In this study it was also found that phosphate concentrations greater than 0.01 M inhibited sclerotia formation regardless of whether nitrogen was supplied as ammonium or nitrate.

Sclerotia tissues in thin sections are observed to have a much different organization than vegetative mycelium. In this study, sclerotia were found to be made up of pseudoparenchymatous tissue. Sclerotia production is probably a morphogenetic change, requiring changes in the enzyme activity. Thus the nitrogen source may be involved with induction and repression at the genetic level for Morchella.

CONCLUSIONS

1. Specific levels of nitrogen, phosphorous, potassium or sucrose do not induce morphogenesis to reproductive growth for Morchella.
2. Neither an increase nor a gradual exhaustion of nutrients induces reproductive growth for Morchella.
3. Ammonium or nitrate salts can both serve as good nitrogen sources for Morchella. The concentrations of the ammonium ion for optimum growth is about 0.01 M. Higher levels of ammonium lead to inhibition of growth, especially at levels greater than 0.05 M. Optimal levels of the nitrate ion are at 0.05 M or higher. Inhibitory effects do not occur at levels of nitrate as high as 0.6 M.
4. When ammonium and nitrate are both present, Morchella utilizes the ammonium preferentially.
5. Growth of Morchella increases as the concentration of potassium, as potassium nitrate, increases to 0.25 M. Levels of potassium nitrate as high as 0.6 M are not inhibitory.
6. Benefits due to the presence of increased amounts of phosphate, supplied as dibasic sodium phosphate and monobasic potassium phosphate, are largely due to buffering effects.
7. Increases the phosphate concentration bring about greater vegetative growth by Morchella, to a concentration of 0.05 M. Higher concentrations are inhibitory.
8. Morchella grows well over a pH range from 3.0 to 6.25, with the best growth observed at pH 5.0.

9. Morchella can grow well on all the sugars tested. Glucose, mannose and sucrose provided for the best growth. The ability to utilize some sugars is related to the supply of minerals and nitrogen.
10. Morchella responds to increasing levels of sucrose, up to 100 g/l with increasing growth.
11. Substances present in potato tubers and Morchella sporocarp tissue promote growth of Morchella mycelium. Morchella sporocarp tissue added to the medium promotes sclerotia formation.
12. The presence of ammonium or levels of phosphate greater than 0.01 M, inhibit sclerotia production by cultures on agar. Sclerotia are produced in high numbers if nitrate salts are the sole nitrogen source.
13. The tested vitamins do not appear to stimulate growth of Morchella significantly. Thiamine, inositol, and nicotinic acid are inhibitory, at the concentrations tested.

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APPENDIX A

Table 3. Standard medium, with buffer.

Component	Concentration
NH_4NO_3	1650.0 mg/l
KNO_3	1900.0
H_3BO_3	6.2
KH_2PO_4	170.0
KI	0.83
Na_2MoO_4	0.025
$CoCl_2 \cdot 6H_2O$	0.025
$CaCl_2 \cdot 2H_2O$	440.0
$MgSO_4 \cdot 7H_2O$	370.0
$MnSO_4 \cdot H_2O$	22.3
$ZnSO_4 \cdot 7H_2O$	8.6
$CuSO_4 \cdot 5H_2O$	0.025
Na_2EDTA	37.35
$FeSO_4 \cdot 7H_2O$	27.85
Thiamine HCl	0.1
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Phosphate Buffer:	
Na_2HPO_4	355.0
KH_2PO_4	6460.0
Sucrose	30 g/l
Agar	10 g/l

APPENDIX B

Table 4. Analysis of variance: Nitrogen tests.

Source of Variation	df	SS	MS	F
Source	3	203346	67782	28.90*
Concentration	4	1138826	284706.50	121.34*
Cells	19	2415342		
Source X Concentration	12	1073170	89430.81	38.13*
Error	69	161850	2345.65	
Total	88	2577192		

*Significant at the .01 level.

Table 5. Analysis of variance: Variable concentration of potassium nitrate.

Source of Variation	df	SS	MS	F
Among Treatments	11	1363660	123969.09	23.38*
Within Treatments	42	222661	5301.45	
Total	53	1586321		

*Significant at the .01 level.

Table 6. Analysis of variance of cultures grown on media containing different concentrations of phosphate ion.

Source of Variation	df	SS	MS	F
Among Treatments	7	3532682.80	504668.97	36.48*
Within Treatments	31	428813.20	13832.67	
Total	38	3961496.00		

*Significant at the .01 level.

Table 7. Analysis of variance of cultures grown on media having different initial pH.

Source of Variation	df	SS	MS	F
Among Treatments	7	1779148.4	254164.05	79.82*
Within Treatments	30	95525.6	3184.19	
Total	37	1874674.0		

*Significant at the .01 level.

Table 8. Analysis of variance of cultures grown with different sugars and the standard, unbuffered medium.

Source of Variation	df	SS	MS	F
Among Treatments	10	1893414.00	189341.40	65.69*
Within Treatments	40	115298.35	2882.46	
Total	50	2108712.35		

*Significant at the .01 level.

Table 9. Analysis of variance of cultures grown with different sugars and increased levels of NPK.

Source of Variation	df	SS	MS	F
Among Treatments	10	3020921.32	302092.13	123.28*
Within Treatments	38	93114.32	2450.38	
Total	48	3114035.64		

*Significant at the .01 level.

Table 10. Analysis of variance of cultures grown on media having different concentrations of sucrose.

Source of Variation	df	SS	MS	F
Among Treatments	4	521695.25	130423.81	27.70*
Within Treatments	18	84763.75	4709.10	
Total	22	606459.00		

*Significant at the .01 level.

Table 11. Analysis of variance of cultures in media containing different vitamins.

Source of Variation	df	SS	MS	F
Among Treatments	10	18588.91	1858.89	3.99*
Within Treatments	43	20035.26	465.94	
Total	53	38624.17		

*Significant at the .01 level.

Table 12. Analysis of variance of cultures on media containing a homogenate of Morchella sporocarps in various concentrations.

Source of Variation	df	SS	MS	F
Among Treatments	4	341277.67	85319.42	148.13*
Within Treatments	18	10367.20	575.96	
Total	22			

*Significant at the .01 level.

Table 13. Analysis of variance of cultures grown on media containing different amounts of an extract from potato tubers.

Source of Variation	df	SS	MS	F
Among Treatments	5	1672761.00	334552.20	79.45*
Within Treatments	22	92644.00	4211.09	
Total	27			

*Significant at the .01 level.

APPENDIX C

Table 14. Significance of differences between treatments in the nitrogen tests, as evaluated by Tukey's omega test.*

	Levels Within Sources				
NH_4NO_3:					
Level	0.10 M	0.001 M	0.05 M	0.005 M	0.01 M
Replications	5	3	3	4	5
Mean, mg	0	132.00	305.00	531.75	666.40
NH_4Tartrate:					
Level	0.10 M	0.05 M	0.001 M	0.005 M	0.01 M
Replications	2	3	5	5	5
Mean, mg	10.50	55.33	226.00	329.20	454.00
NH_4Cl:					
Level	0.001 M	0.10 M	0.05 M	0.005 M	0.01 M
Replications	5	5	5	5	5
Mean, mg	66.40	166.00	202.40	284.60	297.00
$NaNO_3$:					
Level	0.001 M	0.005 M	0.01 M	0.10 M	0.05 M
Replications	5	4	5	5	5
Mean, mg	130.40	183.50	294.80	327.60	348.20

*Treatments underlined by the same line are not significantly different at the .05 level.

Table 15. Significance of differences between treatments in the nitrogen tests, as evaluated by Tukey's omega test.*

Sources Within Levels

0.001 M:				
Source	NH_4Cl	$NaNO_3$	NH_4NO_3	$NH_4Tar.$
Replications	5	5	3	5
Mean, mg	66.40	130.40	132.00	226.00
0.005 M:				
Source	$NaNO_3$	NH_4Cl	$NH_4Tar.$	NH_4NO_3
Replications	4	5	5	4
Mean	183.50	284.60	329.20	531.75
0.01 M:				
Source	$NaNO_3$	NH_4Cl	$NH_4Tar.$	NH_4NO_3
Replications	5	5	5	5
Mean	294.80	297.00	454.00	666.40
0.05 M:				
Source	$NH_4Tar.$	NH_4Cl	NH_4NO_3	$NaNO_3$
Replications	3	5	3	5
Mean, mg	55.33	202.40	305.00	348.20
0.10 M:				
Source	NH_4NO_3	$NH_4Tar.$	NH_4Cl	$NaNO_3$
Replications	5	2	5	5
Mean, mg	0	10.50	166.00	327.60

*Treatments underlined by the same line are not significantly different at the .05 level.

Table 17. Significance of differences between cultures grown with different concentrations of phosphate in the medium, as evaluated by Tukey's omega test.

Conc., M	.0125	0.005	0.20	0.15	0.01	0.10	0.75	0.05
Reps.	4	5	5	5	5	5	5	5
Mean, mg	195.00	387.80	581.00	775.80	846.60	854.20	986.20	1223.20

*Treatments underlined by the same line are not significantly different at the .05 level

Table 16. Significance of differences among cultures grown on media containing different concentrations of potassium nitrate, as evaluated by Tukey's omega test.*

Level	0.01 M	0.05 M	0.10 M	0.15 M	0.30 M	0.20 M
Replications	5	4	5	1	5	4
Mean, mg	207.80	298.25	371.60	446.00	536.20	608.00

(Continued)	0.40 M	0.45 M	0.35 M	0.25 M	0.60 M	0.50 M
	5	5	5	5	5	5
	630.40	642.00	648.00	654.40	663.60	686.80

*Treatments underlined by the same line are not significantly different at the .05 level.

Table 19. Significance of differences between cultures receiving different sugars and the standard, unbuffered medium, as evaluated by Tukey's omega test.*

Sugar	Glu.	Mann.	Suc.	Galac.	Lact.	Rhamn.	Fruct.	Arab.	Glu.A.	Xylose	Gal.A.
Reps.	5	5	5	5	5	5	5	5	5	4	2
Mean, mg	606.60	587.80	337.60	224.00	162.00	156.60	121.60	109.20	69.40	68.75	35.00

*Treatments underlined by the same line are not significantly different at the .05 level.

Table 20. Significance of differences between cultures grown on media having different sugars and with increased levels of NPK, as evaluated by Tukey's omega test.*

Sugar	Mann.	Suc.	Glu.	Fruc.	Galac.	Xylose	Arab.	Lact.	Rhamn.	Gal.A.	Glu.A.
Reps.	5	5	5	5	5	5	5	4	5	2	3
Mean, mg	755.00	674.20	626.40	592.00	391.40	332.60	207.40	185.76	77.60	15.50	15.33

*Treatments underlined by the same line are not significantly different at the .05 level.

Table 18. Significance of differences among cultures on media with variable initial pH, as evaluated by Tukey's omega test.*

pH	2.5	3.0	3.5	4.0	4.5	6.25	5.5	5.0
Replications	5	4	5	5	5	5	5	4
Mean, mg	0	93.00	439.40	485.60	535.80	566.80	577.80	611.75

*Treatments underlined by the same line are not significantly different at the .05 level.

Table 21. Significance of differences between cultures grown on media having different sucrose concentrations, as evaluated by Tukey's omega test.*

Concentration, g/l	5	50	200	150	100
Replications	5	5	4	4	5
Mean, mg	115.60	247.80	327.75	456.50	527.00

*Treatments underlined by the same line are not significantly different at the .05 level.

Table 22. Significance of differences between treatments with potato extract in the media, as evaluated by Tukey's omega test.*

Amount of Extract	0%	5%	75%	10%	50%	25%
Replications	5	5	5	4	5	4
Mean, mg	93.20	524.60	561.80	654.00	671.40	864.00

*Treatments underlined by the same line are not significantly different at the .05 level.

Table 23. Significance of differences between treatments with different amounts of sporocarp homogenate in the media, as evaluated by Tukey's omega test.*

Amount of Homogenate	0.01%	0%	0.10%	1.0%	10.0%
Replications	5	4	5	5	4
Mean, mg	90.20	97.50	97.60	151.40	425.50

*Treatments underlined by the same line are not significantly different at the .05 level.

Table 24. Evaluation of vitamin treatments by Dunnett's test.

Treatment	Replications	Mean, mg	Control - Treatment*
Control	5	31.84	--
Nicotinic acid	5	0.14	31.70
Thiamine	5	0.52	31.32
Inositol	5	5.56	34.28
Biotin	5	29.38	2.46
p-aba	5	40.14	- 8.30
Pyridoxine HCl	5	49.28	-14.84
Ca Pantothenate	5	51.14	-19.30
Riboflavine	5	42.74	-10.90
Folic acid	5	46.30	-16.23
All vitamins	4	32.88	- 1.04

*Minimum difference for significance at the 0.05 level; 38.31 with 5 replications, 41.17 with 4 replications.