FACTORS INFLUENCING GROWTH AND SPORE GERMINATION OF SPECIES OF BOLETUS

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Factors Influencing Growth and Spore Germination of Species of *Boletus*.

by

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A thesis submitted to the University of St. Andrews for the degree of Master of Science.

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D E C L A R A T I O N

I hereby declare that the following thesis is based on a record of work done by me, that the thesis is my own composition, and that it has not previously been presented for a Higher Degree.

The research was carried out in the Department of Botany at St. Salvator's College of the University of St. Andrews under the direction of Professor J.A.Macdonald.

C E R T I F I C A T E

I certify that Margaret L.P.Watson has spent seven terms of research work under my direction, that she has fulfilled the conditions of St. Andrews Ordinance No. 51, and that she is qualified to submit the accompanying thesis in application for the degree of Master of Science.

C A R E E R

After twenty five months of war service in market gardening, I studied for two years at the Midland Agricultural College, gaining the Diploma in Horticulture of University College, Nottingham. Thereafter I matriculated as an ex-service student at St. Andrews University. I graduated in June, 1950, with a Bachelor of Science Ordinary Degree in Botany and Zoology.

For seven years I worked as a Horticultural Adviser, first with the Ministry of Agriculture and Fisheries, and later with the West of Scotland College of Agriculture. For two of those years I was also an Inspector under the Destructive Insects and Pests Acts and Orders.

Since 1961 I have been teaching Biology at St. Leonards and St. Katharines Schools, St. Andrews. I matriculated at the University of St. Andrews in November, 1967, and was admitted as a part-time research student under St. Andrews Ordinance No. 51 to undertake the research work presented here for the Degree of Master of Science.

A C K N O W L E D G E M E N T S

I wish to express my thanks to Professor J.A. Macdonald, my supervisor who suggested the problem, for his help, encouragement and valuable criticism throughout this work.

I am also indebted to Mr.R.L.Constable of the Statistics Department of St. Andrews University for analysing statistically the data in Part 1, Sections 3 and 4, of this thesis, and to the technical staff of the Department of Botany, notably Mr.J.Hill for help with the preparation of culture media and Mr.R. Stephenson for taking the photographs.

I should also like to take the opportunity to express my appreciation to Miss J.S.A.Macaulay and the Council of St. Leonards and St. Katharines Schools, St. Andrews, for granting me leave of absence for four months at the beginning of 1970, and to Professor Macdonald for appointing me as his research assistant during that time.

C O N T E N T S

P A R T 2

I N T R O D U C T I O N

Studies in the genus Boletus have been in progress in the Botany Department of St. Andrews University for a considerable number of years. Several species can be found nearby at Tentsmuir, Fife, in an area bordering the Nature Reserve. Some of them grow in the coniferous woods, whilst others grow at quite a distance from trees in the regions of the sand dunes. Much of the work being undertaken is directed towards investigating the reasons for the species growing in such diverse habitats, and it is hoped that the researches recorded here may contribute something useful to the work of the Department.

The genus Boletus is included in the class Basidiomycetidae, which is the most advanced of all the fungal classes. In the sub-class Homobasidiomycetidae Ainsworth (1961) includes the Boleti in the order Agaricales.

The order Agaricales includes the fungi whose fruiting bodies are usually spoken of as mushrooms and toadstools, and another group of large, fleshy fungi

called the boletes. The mushrooms and toadstools bear their basidia at the surface of gills or plates which are generally produced on the underside of fleshy, umbrella-like sporophores. In the boletes, the basidia line the inside of deep tubes on the underside of putrescent, mushroom-like sporophores.

The genus Boletus is world wide in its distribution, being found in Europe, North America, Australia and Africa in areas where rainfall is adequate for the development of most mushrooms.

The New Check List of British Agarics and Boleti by Dennis, Orton and Hora (1960) gives forty five British species of Boletus. The nomenclature adopted by them is used throughout this work. Watling (1970 b) brought out a relevant section of a new British Fungus Flora just before this thesis was completed and too late for certain changes of nomenclature which he has introduced to be embodied in the work.

So far twelve species have been identified positively at Tentsmuir. Dr. E.G.Duncan (verbal communic-

ation names these as:-

Boletus badius, Fr.1828; B.bovinus, L. ex Fr.1821; B.chrysenteron Bull. ex St.Amans,1821; B.elegans, Schum. ex Fr.1838; B.erythropus (Fr. ex Fr.) Secr.,1833; B.luteus L. ex Fr.,1821; B.piperatus Bull. ex Fr.,1821; B.reticulatus (Schaeff.) Boud.,1876; B.scaber Bull. ex Fr.,1821; B.subtomentosus L. ex Fr.,1821; B.variegatus Sow. ex Fr.,1821; B.testaceoscaber Secr.,1833 (B.versipellis Fr., and Hök,1836, s. auct. brit., Fr. and Hök p.p)

The number of species being studied was originally restricted to five, namely B.badius, B.bovinus, B.edulis, B.luteus and B.variegatus. As this work was started at the end of a growing season (November 1967), cultures of these five species, which it was believed at that time could be found at Tentsmuir, were obtained from the Centraalbureau voor Schimmelcultures at Baarn in the Netherlands, in order to provide stocks of cultures for nitrogen and carbon utilisation investigations, to determine suitable growth media for the different species and to study the growth characteristics of their mycelia.

Between July and October 1970, more than thirty visits were made to Tentsmuir in search of B.bovinus, but at no time did the writer find it. In conversation, Dr.E.G.Duncan expressed the view that the fructifications of this species would not be found that season because it has an irregular fruiting cycle and fruit bodies had been collected in 1967.

Dr.R.Watling (verbal communication) stated that the single fruit body found at Tentsmuir and identified as B.edulis was incorrectly named. In his view, B.edulis does not grow so far north and the specimen in question was probably a sport of B. reticulatus.

Thus the investigation of both B.bovinus and B.edulis was limited to the study of growth characteristics of their mycelium, and nitrogen and carbon tests carried out with cultures obtained from the Netherlands in 1968.

The mycelium of all boletes grows in the soil. Trappe (1962) lists eighty eight species as forming mycorrhizal associations with trees. Of the five

species presently under consideration, none is specific to one higher plant species. All of them can be found in association with conifers and, apart from B.edulis, with oak. All three which occur at Tentsmuir were found growing under Pinus sylvestris.

The twofold objective of this research was to isolate mycelium from freshly harvested fruit bodies in order to carry out tests on the nitrogen and carbon utilisation of these species and to attempt to find out the conditions necessary for the germination of Boletus spores. The extensive studies of Fries (1941, 1943, 1949, 1966) have show the latter to be a difficult problem. The nutrition tests were planned as a first step in studying the relationship between roots of pine and the fungi concerned, which are all mycorrhizal.

PART 1

SECTION 1

Materials and Methods.

Materials.

Cultures of B.badius, B.luteus and B.variegatus were prepared from fruit bodies collected at Tentsmuir in 1970.

Cultures of five species of Boletus were obtained from the Centraalbureau voor Schimmelcultures at Baarn in the Netherlands on 13 June 1968 under the following references:-

Xerocomus badius (Fr.) Kühner ex Gilbert,
Syn. Boletus badius Fr. 445.64 Kuhn & Co. 31.5.1968
Suillus bovinus (L.ex Fr.) O.Kuntze 113.31 BIHM 31.5.1968
Boletus edulis Bull. ex Fr. 130.53, Loos 31.5.1968
Suillus luteus (L. ex Fr.) S.F.Gray 136.36
fr. Pinus sylvestris, F.M.Muller 31.5.1968
Suillus variegatus (Sow. ex Fr.) O.Kuntze 117.42,
Melin 31.5.1968.

Unless otherwise stated, the chemicals used were supplied by British Drug Houses Limited and were of Analar Grade.

General Methods.

1. Sterilisation of Glassware

Petri plates, test tubes, watch glasses and slides (the latter wrapped individually in aluminium foil and placed in petri plates) were sterilised with dry heat at 180°C for two hours. The Erlenmeyer flasks were washed with chromic acid, rinsed three times in tap water and then once in distilled water, plugged with non-absorbent cotton wool and capped with aluminium foil before sterilising by autoclaving at 15 lb pressure per square inch for 15 minutes.

2. Preparation and Sterilisation of Culture Media.

The recipes for the culture media are detailed in the Appendices. Unless otherwise stated in the recipe, all the culture media were sterilised by autoclaving at 15 lb pressure per square inch for 15 minutes, except the nitrogen compounds and the sugar solutions. Each of these was dissolved in

distilled water and sterilised by steaming at 100°C for 30 minutes at 24 hour intervals on each of three successive days.

For the most part, the stock cultures were maintained on malt extract agar. B.variegatus from Baarn was contaminated with bacteria. This was checked by repeated subculturing on Martin's Medium (1951) before returning it to malt extract agar. During a phase when contamination by Penicillium spp. was evident, all stocks were transferred to a selective medium for the isolation of Basidiomycetes, Russell (1956).

Growth rates of mycelium for all species were estimated using petri plate cultures with malt extract agar as the culture medium. Cultures were grown in darkness at 18°C.

3. Fruit Bodies for the Preparation of Tissue Cultures.

Fruit bodies of B.badius, B.luteus and B.variegatus were picked and placed singly in previously unused polythene bags. Within three hours of collecting, they were taken to the laboratory. Tissue cultures

were prepared by a process which combined the method of Pantidou (1961) with the culture medium of Ferry and Das (1968). Flamed forceps were used to remove portions of the pileus at the point where it joins the stipe. This tissue was partially buried in the culture medium which was sloped in McCartney bottles. These were incubated in the dark at 21°C and mycelial growth was observed within 10-16 days.

4. Culture and Mounting of Mycelium for Microscopic Examination.

Petri plates containing Martin's Medium were inoculated at the edge with the mycelium, this medium being chosen to check any bacterial contamination. Sterilised malt extract agar was poured onto sterile slides which were inserted into the plates. When the mycelium grew onto the slides, it was fixed with acetic alcohol. The slides were left in fixative for 15-30 minutes. They were rinsed thoroughly in absolute alcohol. Finally they were mounted in Gurr's Neutral Mounting Medium.

SECTION 2

Description of Species

Habitat in this enquiry the fruit bodies were always found in mature stands of P.sylvestris. B.badius and B.luteus were always found in grass. B.variegatus was usually found in grass, but occasionally on sandy soil with no cover crop. Watling (1970b) states that B.badius is common under P.sylvestris but may continue to fruit long after conifers have disappeared from the area; that B. bovinus may be found in coniferous plantations or with isolated pines; that B.edulis may be found in mixed, broad-leaved or coniferous woodland.

B.badius

Fruit body chestnut brown, 5-7 cm, convex, smooth and shiny when dry; Tubes white, becoming yellowish-green; Pores turning blue-green when bruised; Stipe striate and pale brown with a whitish base (Fig.1).

Spores were not collected. Watling (1970b) describes them as olivaceous snuff brown; 13-15/4.5-5.5µm; Subfusiform in side view, ellipsoid in face view.

Mycelium <u>macroscopic characters</u>

Rate of growth 5-8 cm in a month, off white, tufted (Plate 1).

<u>microscopic characters</u> hyphae hyaline, interwoven 5.5-9µm wide. Clamp connections not seen.

<u>B.bovinus</u>

Fruit bodies of this species were not found, but the distinguishing characters given by Wakefield and Dennis (1950) and Lange and Hora (1963) are Cap deep buff or dull reddy brown, 4-8 cm, convex to flat with margin whitish or pale at first; Tubes slightly decurrent, greeny-brown; Pores large, compound; Stipe short, tapering downwards. Spores were not collected. They are described as ochraceous sienna, subfusiform-ellipsoid, 8-10/2.5-3.5µm, (Watling, 1970b).

Mycelium <u>macroscopic characters</u>

Rate of growth 2-5 cm in a month, upper surface pink-buff, lower surface cinnamon, yellow exudate from hyphae observed in spring (Plate 2).

<u>microscopic characters</u> hyphae hyaline, twisted, 4.5-6µm wide.

Clamp Connections not seen.

<u>B.edulis</u>

Fruit bodies of this species were not found, but the distinguishing characters given by Wakefield and Dennis (1950) and Lange and Hora (1963) are Cap brownish, smooth, 6-20 cm, hemispherical to convex; Tubes white, not bruising bluish; Stipe swollen, white network confined to the upper part. Spores were not collected. They are described as olivaceous snuff-brown, 14-17/4.5-5.5µm, (Watling, 1970b).

Mycelium <u>macroscopic characters</u>

Rate of growth 5-7 cm in a month, white (Plate 3).

<u>microscopic characters</u> hyphae hyaline,

cell ends slightly tapered, 5-10μm wide. Clamp Connections not seen.

B.luteus

Fruit body brown to brownish-yellow with a slight tinge of purple, 5-8 cm, convex, glutinous becoming shiny when dry; Tubes, at first covered by a white membranous veil, pale yellow becoming dirty yellow-green; Stipe yellow, granular, above a cream coloured ring darkening to purple, white or brownish below (Fig.2). Spores pale yellowy-fawn spindle-shaped, 7-10/3-3.5μm.

Mycelium macroscopic characters

Rate of growth 4-6 cm in a month, furry, upper surface pale cinnamon pink, lower surface yellowy-brown (Plate 4).

microscopic characters hyphae hyaline, 2-3.5μm wide, branched. Clamp Connections not seen.

B.variegatus

Fruit body dull yellow-brown with darker

brown scales, 6-12 cm, convex at first, flat later, sometimes slimy but dries to a dull surface; Tubes dark buff, bluish when bruised; Stipe stout, yellow above merging to brown below (Fig.3). Spores were not collected. They are described as snuff brown, 9-11/3-4μm, subfusoid elongate to ellipsoid in side view, (Watling, 1970b).

Mycelium <u>macroscopic characters</u>

Rate of growth 5-8 cm in a month, off-white, tufted (Plate 5).

<u>microscopic characters</u> hyphae hyaline, 5.5-9μm wide. Clamp Connections not seen.

Pantidou (1961) says of the microscopic characters, "most species of Boletaceae show a uniformity which is annoying for the taxonomists". In describing several species, including <u>B.luteus</u>, she records the fact that she has seen no clamp connections. Pantidou and Groves (1966) looked at the same species again and did see occasional clamp connections. They are also recorded for <u>B.luteus</u> by Mosca and Fontana (1965). Melin (1923) in his extensive investigations never observed clamp connections in <u>B.badius</u> and seldom

in B.luteus. He found that in B.variegatus they occurred relatively abundantly though irregularly. For the five species described above, clamp connections were not seen by Watling (1970 b).

Period over which fruit bodies were found.

B.badius	3rd. week August - 4th week September, 1970
B.luteus	1st. week June - 1st. week November, 1968
	3rd. week September - 4th. week October, 1969
	1st. week June - 4th week October, 1970
B.variegatus	3rd. week August - 4th. week September, 1970

SECTION 3

Utilisation of Nitrogen Sources.

This experiment was carried out to determine which compounds could be utilised as sources of nitrogen by B.badius, B.bovinus, B.edulis, B.luteus and B.variegatus. In the first instance, the cultures obtained from the Centraalbureau voor Schimmelcultures at Baarn were used. A year later (February - March, 1971), the experiment was repeated using mycelium isolated from fruit bodies of B.badius and B.variegatus harvested at Tentsmuir in the summer of 1970.

Although cultures were built up of B.badius, B.variegatus and B.luteus from Tentsmuir, the last was not used because its rate of growth was found to be so very much slower than that of the other two species. This made it impossible to produce sufficient mycelium of a comparable age and vigour. The extreme differences in growth rate are illustrated in Plates 1-5.

The faster growing species, i.e. B.badius and

B.edulis, were grown for one week on malt extract agar at 22°C, whilst the other three species had to be grown for three weeks before they increased sufficiently to be used as sources of inoculum.

Potassium nitrate, potassium nitrite, ammonium tartrate, alanine, asparagine, glycine and methionine were incorporated separately in the basal medium suggested by Lilly and Barnett (1951), (Table 1). The quantity of each nitrogen compound used was calculated to contain a weight of nitrogen equivalent to that contained in 2 gm asparagine.

The potassium nitrate, ammonium tartrate and glycine were all Analar grade reagents. The other nitrogen sources were laboratory grade reagents, and all of them were supplied by British Drug Houses Ltd.. Analar grade nitrogen sources were used where these were available in an attempt to reduce the amount of chemically combined nitrogen which might be present as an impurity in the medium.

Although Cochrane (1958) pointed out that urea is a utilisable nitrogen source, only experiments

using cold sterilised urea are valid, since it breaks down to ammonia on autoclaving. McKay (1968) tried to overcome this difficulty by steam sterilising at 100°C. Her results suggest that even so the urea was broken down. Because of these findings, and because the facilities available to the writer did not permit cold sterilisation, urea was not used as a nitrogen source.

Potassium nitrite was included in an attempt to learn something about the manner in which nitrates become available for Basidiomycetes, and to see if, in fact, there are any species of Boletus which can utilise nitrites but not nitrates.

The experiment was set up in duplicate and the media were dispensed in 25 ml quantities in previously sterilised 100 ml Erlenmeyer flasks. The pH of all media was adjusted to 6.0 before sterilisation.

In this, as in all quantitative experiments in this investigation, as nearly as possible equal-sized pieces of inoculum were used. A 5 mm square was outlined on a piece of graph paper. Each petri plate

was superimposed on the graph paper so that the growing edge of the colony coincided with one side of the square. The inoculum was cut out 5 mm square and approximately 2 mm deep.

In each case the inoculum was transferred aseptically to the liquid medium and allowed to float so that the fungus could get free oxygen. Incubation in a culture room at 22°C, in the dark followed for four weeks.

For B.badius each piece of inoculum floated during the entire experiment. Growth was rapid with production of a snowy white mycelium completely covering the surface of the medium and growing to a height of 5 mm above it. Within two weeks a purple tinge was evident in the liquid immediately below the mycelium. After three weeks the mycelial mat developed yellow and cinnamon pigments.

B.edulis was a second species in which each piece of inoculum floated during the entire experiment. Again, the mycelial mat completely covered the liquid and grew to a height of 5 mm above it. By the third

week the smooth surface of the mycelium began to have a gritty, spikey appearance as a tufted habit of growth began to develop. At all times the mycelium was snowy white and the liquid medium was cloudy.

Every inoculum of B.bovinus sank within twenty four hours of setting up the experiment. No growth was evident till the end of the second week when very short, densely crowded hyphae were observed growing all through the liquid, but restricted to under the surface. These tended to break off and, at the end of four weeks, the culture medium was found to contain granular-looking fragments of pinky-buff mycelium.

B.luteus appeared to behave in the same manner as B.bovinus, with every inoculum sinking. As the short, dense hyphae broke away, they remained submerged. The liquid took on a granular, cloudy appearance. The hyphae were fawn coloured.

In the case of B.variegatus, again every inoculum sank and growth appeared to be slow. As the fragmented hyphae grew, some of them floated to the surface giving the slightly cloudy medium a curdled

appearance. The hyphae were white.

B.badius and B.edulis, the two species which floated throughout the experiment, showed thereby that they are strongly aerobic, whilst B.bovinus and B.luteus, which sank, showed themselves to be strongly anaerobic. B.variegatus, with its surface and submerged mycelium, supported a dual role. This leads to the speculation that individual species may in nature be linked with specific soil types, i.e. with different degrees of aeration of specific soil types or with different heights of water table. For example, different species may vary in their ability to grow successfully in water logged soil. The condition of the soil may be as vital a factor as the presence of the conifer in determining the establishment or otherwise of the mycorrhiza relationship.

At the end of four weeks the mycelial mats were separated from the culture media by filtering through Whatman filter papers (which had been weighed) and were dried to constant weight at 50°C. The mean dry weights of the mycelia and the pHs of the filt-

rates are shown in Tables 2 and 3. (Appendices).

Results

The results for all the fungi were taken from the same experiment which should rule out any differences due to variation in the basal medium or in the age and vigour of the inoculum. For all species each weight was calculated as a percentage of the control and the results are shown in the form of histograms. (Appendices).

The results presented in Tables 2 and 3 indicate that no two species grew equally well on the same sources of nitrogen.

With the Dutch cultures, the greatest weights of mycelium were produced as follows:-

B.badius and *B.bovinus* on glycine;
B.edulis on ammonium tartrate;
B.luteus on potassium nitrate and
B.variegatus on alanine.

Again with the Dutch cultures, the lowest weights were found to be:-

B.badius and B.edulis on methionine;

B.bovinus on potassium nitrate;

B.luteus on ammonium tartrate and

B.variegatus on asparagine.

With the exception of B.luteus growing on ammonium tartrate, alanine and asparagine, and B.variegatus on asparagine, all the species produced a greater weight of mycelium on the media containing nitrogen than they did on the control medium which had no added nitrogen.

The pH values of the culture media changed during growth as shown in Tables 2 and 3. The values for the filtrates from the nitrate and nitrite media were higher than the initial pH. Those from the ammonium media fell to a much lower figure. The pH values from the media containing organic nitrogen fell to figures similar to those of the ammonium medium in fourteen cases. The value for B.bovinus on alanine dropped, but not so low, whilst the pH rose in the remaining four, namely B.badius on glycine and B.edulis

on alanine, asparagine and glycine.

In assessing the utilisation of the seven nitrogen sources by the five species of Boletus obtained from Baarn, points were allocated to each source on the basis that the one which produced the greatest weight of mycelial growth for any species was given seven points; the source which produced the next greatest weight was given six points, and so on in descending order to the source which yielded the lightest weight which was given one point.

Using this method, the total number of points scored by each nitrogen source was as follows:-

glycine	29
potassium nitrite	23
potassium nitrate	21
ammonium tartrate	20
alanine	19
methionine	16
asparagine	15

This shows that, apart from organic nitrogen in the form of glycine, the inorganic salts and ammonium

nitrogen compounds produced greater weights of mycelium than the organic sources did. These differences are interesting and are statistically significant, especially for glycine and potassium nitrite.

In making the statistical calculations, use was made of a Gausian Curve. By this method, a difference is not considered to be significant until it comes down to less than 5% chance. Thereafter, the lower it goes, the more highly significant it becomes.

For the experiment under consideration, the main variation is caused by the control which is significantly less (as might be expected) than all the others. There is apparently no statistical difference between inorganic and organic nitrogen, but glycine has given significantly higher values (at 5% level). However this appears to be mainly due to the very high value with B.badius and should not be regarded as more than a chance occurrence.

With the Tentsmuir cultures, for B.badius the results were similar to those recorded in 1970, using the Dutch cultures. Apart from the fact that aspar-

agine and glycine changed places, i.e. asparagine produced the greater weight of mycelium, the weights recorded followed the same order, and were almost identical.

Nitrogen Source	Tentsmuir Cultures	Dutch Cultures
Asparagine	199.15 mg	192.35 mg
Glycine	195.95 mg	195.3 mg
Alanine	165.95 mg	168.5 mg
Pot. nitrate	154.9 mg	155.4 mg
Amm. tartrate	141.15 mg	137.75 mg
Pot. nitrite	104.7 mg	100.65 mg
Methionine	100.1 mg	99.3 mg

There was a marked difference in the weights recorded for B.variegatus. Apart from potassium nitrite and methionine, the weights of the Tentsmuir cultures were higher than the Dutch ones.

Nitrogen Source	Tentsmuir Cultures	Dutch Cultures
Glycine	145.4 mg	73.15 mg
Alanine	135.35 mg	84.6 mg
Amm. tartrate	131.75 mg	66.7 mg
Pot. nitrate	118.75 mg	74.3 mg

Nitrogen Source	Tentsmuir Cultures	Dutch Cultures
Asparagine	113.95 mg	58.4 mg
Pot. nitrite	61.55 mg	76.9 mg
Methionine	34.3 mg	73.15 mg

The variation in the B.variegatus figures for 1971 tends to cancel out any statistical conclusion for that species and destroys the pattern of results accruing from the 1970 figures.

It is noted that the B.badius figures compare very closely with those for the Dutch cultures.

When the statistical method which employs the Gausian Curve is applied to the Tentsmuir cultures, there is a highly significant difference ($< 1\%$) between the species and between the nitrogen sources.

Discussion

The Boletus species under consideration were able to utilise organic and ammonium nitrogen, apart from B.luteus with asparagine, alanine and ammonium tartrate and B.variegatus (Dutch) with asparagine. It has been recorded by other workers that asparagine and glycine are particularly good sources of nitrogen for many Basidiomycetes. Fries (1955) showed experimentally that asparagine was a good nitrogen source for most of the eighteen Coprinus species which he studied. Cochrane (1958) states that most investigators have agreed that glycine and asparagine are the most likely nitrogen sources to support growth. The writer's results bear out these claims for glycine, but not for asparagine, which was poorly utilised by B.bovinus and B.edulis and was not utilised by B.luteus or B.variegatus (Dutch).

B.edulis, B.luteus and B.variegatus did well with potassium nitrite but, apart from B.luteus and B.variegatus (Tentsmuir), less well with potassium nitrate. The results recorded by many workers show that, for Basidiomycetes in general, growth is slow

on nitrate nitrogen. Melin (1953) showed that only a few of the mycorrhiza-formers which he had investigated could utilise nitrates. Lilly and Barnett (1951) published a list of nitrate utilising fungi but it contained only four Basidiomycetes, none of them Boletus. Lindeberg (1944), working with Marasmius, found that only one out of thirteen species could utilise nitrates. Norkrans (1950) found that Tricholoma nudum was the only one of eight species of Tricholoma studied which could utilise nitrate nitrogen. Garrett (1953) and Dos Santos (1963) have shown that Armillaria mellea was not able to utilise nitrate nitrogen.

The need for some Basidiomycetes to reduce nitrates to nitrites before they are utilised is suggested by the facts that, in the experiment presently under consideration, B.bovinus, B.edulis and B.variegatus produced greater weights of mycelium on potassium nitrite than on potassium nitrate. Placing the weights obtained with all the nitrogen sources in order from the greatest to the least, then the positions of potassium nitrite and potassium nitrate are as follows:-

Fungus	Potassium nitrite	Potassium nitrate
B.luteus	2nd	1st
B.variegatus	2nd	3rd
B.edulis	3rd	4th
B.bovinus	4th	7th
B.badius	6th	4th

Hacskaylo, Lilly and Barnett (1954) found among the fourteen wood-destroying Basidiomycetes which they tested that only a few used nitrates, and that very poorly. Only Polyporous distortus grew well on nitrate, though it did so very slowly. (Dry weight of mycelium produced on KNO_3 = 2 mg after 13 days; 4 mg after 21 days and 164 mg after 40 days). These workers have pointed out that the slow utilisation of nitrogen is exaggerated in some Basidiomycetes when the experiments are of short duration. This is perhaps because it is necessary to reduce the nitrates to nitrites before utilising them. This reduction is evidently a slow process, because when their experiments were allowed to run for longer, nitrates were shown to be utilised by fungi in which this was not apparent before.

With regard to the behaviour of the pH values during the course of this experiment, the values of the pH from the nitrate medium tended to rise to higher values whilst those from the ammonium medium tended to fall. Similar movements are recorded, without explanation, by Hacskaylo, Lilly and Barnett (1954).

The effect of the fungus on the molecule of the compounds was examined to assess what could have been the cause of the changes in pH. The writer has been unable to produce an explanation which is generally satisfactory.

SECTION 4

Utilisation of Carbon Sources.

This experiment was set up to study the utilisation of several sources of carbon by B.badius, B.bovinus, B.edulis, B.luteus and B.variegatus. As with the experiment on the utilisation of nitrogen sources, the Dutch cultures were used in the first instance and a year later (February-March 1971) the experiment was repeated using mycelium isolated from fruit bodies of B.badius and B.variegatus harvested at Tentsmuir in the summer of 1970.

The faster growing species, i.e. B.badius and B.edulis, were grown for one week on malt extract agar at 22°C, whilst the other three species had to be grown for three weeks before the mycelium was large enough to be used as a source of inoculum.

Five carbohydrates supplied by B.D.H. were chosen for the experiment, namely glucose and fructose (monosaccharides), sucrose and maltose (disaccharides) and soluble starch (a polysaccharide). The glucose

and sucrose were Analar grade. The fructose and starch were laboratory grade reagents. The fructose was laevulose, dextrose free.

These five sources of carbon were incorporated separately in the basal medium suggested by Lilly and Barnett (1951), (Appendices), at the rate of 10 g carbon source per litre. As this basal medium contains asparagine, at the rate of 2 g per litre, two sources of carbon are available in each nutrient medium. Thus the experimental results must be the measurement of the additional growth obtained from supplying a second carbon compound in the form of a carbohydrate.

The experiment was set up in duplicate, in the manner described in the nitrogen utilisation experiment. As the carbon and nitrogen sources were sterilised separately from the rest of the culture medium and from each other, the pH of each constituent part was corrected to 6.0 before sterilising.

Incubation in a culture room at 22°C, in the dark, lasted for four weeks.

The visible appearance of the fungi during the period of growth in no way differed from that already described in the nitrogen utilisation experiment in Section 3.

At the end of four weeks the mycelial mats were separated from the culture media by filtering through Whatman filter papers (which had been weighed) and were dried to constant weight at 50°C. The mean dry weights of the mycelia and the pHs of the filtrates are shown in Tables 4 and 5.

Results

The results for all the fungi were taken from the same experiment which should rule out the possibility of differences due to any variation in the basal medium or in the age and vigour of the inoculum. For all species each weight was calculated as a percentage of the control and the results are shown in the form of histograms. (Appendices).

With the Dutch cultures the greatest weights

of mycelium were produced as follows:-

B.badius and B.edulis on glucose;

B.bovinus, B.luteus and B.variegatus on starch.

Again with the Dutch cultures, the lowest weights of mycelium were found to be:-

B.badius and B.variegatus on maltose;

B.bovinus, B.edulis and B.luteus on fructose.

As in the nitrogen utilisation experiment, points were allocated in an attempt to arrive at an order of preference for the carbon sources. In this instance the greatest weight of mycelial growth for any species scored five points, the second greatest weight scored four points and the lowest one point.

Using this method, the total number of points for each carbon source was as follows:-

starch	23
sucrose	19
glucose	14
maltose	10
fructose	9

From the statistical point of view (using the Gausian Curve calculation) the figures in Tables 4 and 5 show that highly significant (<1%) differences occur between species and between carbon sources. Starch gives greater weights than the other sources, except in the case of B.edulis where sucrose and glucose are perhaps more effective.

With the Tentsmuir cultures, for B.badius the results were similar to those recorded in 1970, and the weights of mycelium were recorded in the same order as for the Dutch cultures:-

Carbon Source	Tentsmuir Cultures	Dutch Cultures
Starch	191.65 mg	211.6 mg
Glucose	159.05 mg	160.3 mg
Sucrose	145.3 mg	145.6 mg
Fructose	144.3 mg	143.85 mg
Maltose	123.65 mg	123.3 mg

For B.variegatus from Tentsmuir, apart from the fact that the second greatest weight of mycelium for cultures from both sources was recorded for sucrose, the order was quite changed. The weights of mycelium

were all higher this time, apart from that for starch.

Carbon Source	Tentsmuir Cultures	Dutch Cultures
Maltose	191.9 mg	63.8 mg
Sucrose	176.1 mg	91.6 mg
Starch	174.05 mg	350.75 mg
Fructose	140.4 mg	84.1 mg
Glucose	119.3 mg	83.8 mg

Discussion

Apart from some work carried out by Norkrans (1950a), very few investigations appear to have been made into the utilisation of carbon by mycorrhizal Basidiomycetes. The most extensive work on the utilisation of carbon by Boletus spp. is that of Ferry and Das (1968).

These workers used four species of Boletus, three of which were ones chosen by the writer, namely B.bovinus, B.luteus and B.variegatus. Ferry and Das found invariably that glucose was the best carbon source for all four species and that fructose was

generally poor. This is not in accordance with the writer's results with regard to either experiment. With the Dutch cultures and B.badius from Tentsmuir starch came first with glucose second. With B.variegatus (Tentsmuir) maltose came first and glucose was third.

Of the disaccharides tested by Ferry and Das, sucrose and maltose ranged from good to poor. The writer's results were similar, with higher weights of mycelium always being recorded for sucrose than for maltose (apart from B.variegatus (Tentsmuir)). One can only speculate as to the extent to which any differences between the results recorded by Ferry and Das and by the writer can be attributed to the fact that, in the latter case, the experiments ran for three times as long.

Melin (1925) investigated the utilisation of polysaccharides, including starch (but does not state its nature), by Boletus and found that the growth on this compound was slight. This is in marked contrast with the writer's experience, where the greatest weights of mycelium were recorded on soluble

starch for all species except B.edulis and B.variegatus (Tentsmuir). Melin notes that the growth rate is affected by the age of the cultures when used for inoculation. He found that a greatly increased growth rate resulted from inocula which had been leached in sterile water. Under these circumstances he believed that the inocula contained less staling products and that these products were responsible for a decrease in vigour of mycelia.

How (1940) examined the utilisation by B.elegans of the five carbon sources used by the writer. His results showed glucose to be the best carbon source, but recorded good growth from the other sources, including starch. Although he carried out no tests with cellulose or ligno-cellulose, he suggests that, in a forest soil, the amounts of starch and sugars available for a slow-growing organism such as B.elegans in competition with bacteria must be practically negligible. (He made no mention of "carbohydrate" or "sugar" fungi as competitors with Boletus). From this he argues that it is safe to infer from pure culture work that Boletus can use simple carbon compounds if available in the soil,

but it would not be proper to conclude that inability to utilise more complex substances in pure culture will also show itself in the soil. Harley (1959), on the other hand, points out that phenolic products in the humus can inhibit the development of fungi in their natural environment.

As far as the writer could discover, no work has been published on the changes in pH which take place during the utilisation of carbon by species of Boletus. The figures recorded in Tables 4 and 5 have been studied, but no pattern of change emerges for which an explanation can be offered.

Comparison between 1970 and 1971 Experiments

When considering the results from both the utilisation of nitrogen and the utilisation of carbon experiments, the most notable fact is the closeness in the figures recorded for B.badius in both years and the variation in the figures recorded for B.variegatus.

It was noted at the outset that the culture of B.variegatus from Baarn showed a tendency to bacterial contamination which was not evident in the Tentsmuir culture. The possible presence of bacteria in the 1970 experiment may account for the lower weights recorded. The bacteria could have been utilising the culture medium directly or by parasitising the fungus mycelium. A large part of the weight of the bacteria would not be recorded because they would not be retained with the mycelium by the filter paper. As this possibility was not realised in 1970, the filtrates were not examined for the presence of bacteria.

The B.variegatus culture from Baarn was originally obtained by the Centraalbureau voor Schimmelcultures in 1942. Personal correspondence revealed that their cultures are maintained on cherry agar, a medium which would not be expected to contribute any vitamin B. Melin (1948) has demonstrated that B.variegatus loses its capacity for synthesising "vital substances" when it is deprived of vitaminB. Although biotin and thiamin were both supplied in the writer's experiment, the Dutch culture of B.variegatus could have been less able to utilise vitamin B than the more recently

isolated Tentsmuir culture and might, in consequence, have been less well equipped to develop in the nutrient solution.

There is also the possibility that the two cultures of B.variegatus used are representative of separate strains of the fungus. From the differences in their results, Ferry and Das (1968) suggest that isolates should be obtained from many widely separated geographical areas in order to obtain a representative sample of any species. They also point out that there is a need to employ more than a single isolate from each location to represent a species.

Explanation for the close agreement of the figures for B.badius from Baarn and from Tentsmuir is less easy to suggest. The chance of such precise agreement between figures for two experiments carried out a year apart and employing cultures of B.badius of different origin is in the region of one in ten thousand. Certainly these results speak well for the culture room in which the experiments were conducted, for they indicate that exactly similar conditions for temperature and ventillation must

have prevailed. It also suggests that all the techniques employed in setting up the experiments were identical. But it is very noteworthy that in B.badius, in contrast to B.variegatus, there is so little difference in the behaviour of two mycelia which are of different ecological origin and have been in culture for different lengths of time.

In order to resolve these points, much could be learned by freeing the B.variegatus (Dutch) culture from bacterial contamination and then repeating the experiment with the Dutch and Tentsmuir cultures simultaneously.

PART 2

SECTION 1

Materials and Methods.

Materials.

All the spores of Boletus luteus were collected from Tentsmuir, Fife, in an area bordering the Nature Reserve, in 1967, 1968, 1969 and 1970.

Seeds of Pinus sylvestris were obtained from the Forestry Commission (Research) in Edinburgh. Seeds were collected from Tentsmuir in September 1968 and 1969, but they did not germinate. Dallimore and Jackson (1923) point out that, for this species, good seed years only occur at irregular intervals.

General Methods.

1. Harvesting and Storing of Spores.

Fruit bodies of B.luteus were picked and placed singly in previously unused polythene bags. Within

three hours of collecting, they were taken to the laboratory where each fruit body was placed pore-side down over a petri plate containing malt extract agar and covered with a beaker to keep off draughts. After two hours, by which time the agar was seen to be covered with a layer of spores, strips of agar $1\frac{1}{2}$ X $\frac{1}{2}$ inch were cut from the plates with a flamed scalpel and placed on malt agar slopes in McCartney bottles. These were held in a refrigerator at 10°C until they were needed.

2. Preparation and Sterilisation of Fruit Body Extract.

Immediately after the spores had been shed, the fruit bodies, many of them containing maggots, were minced. The mince was mixed with its own volume of sterile distilled water and was shaken vigorously until all lumps were removed and the water was completely incorporated. This mixture was heated to boiling point and was allowed to simmer for thirty minutes. After it had cooled, the fruit body extract was obtained by straining the mixture through coarse, unbleached calico. As the extract was thick and glutinous, it was shaken up with its own volume of sterile distilled water before being sterilised by

steaming at 100°C for thirty minutes on each of three successive days.

3. Stratification and Surface Sterilisation of Pine Seeds.

In order to ensure a uniform rate of germination, the pine seeds were stratified for thirty eight days using the method of O'Rourke (1964). (A trial set up in 1969 had shown thirty eight days to be the optimum time to obtain the maximum germination of seeds of P.sylvestris). The seeds were mixed with two to three times their own volume of moist, but not wet, sand contained in a previously unused polythene bag. They were placed in a refrigerator at 10°C during the whole of the stratification period. The sand had been autoclaved for four hours at 15 lb pressure.

Wilson's (1915) method was used for surface sterilising the seeds. 10 gm calcium hypochlorite was added to 140 ml distilled water. This was shaken vigorously for a few minutes and then filtered. The clear filtrate was used for sterilising the seeds. The number of seeds required was placed in a small

test tube and the clear filtrate was added. The tube was shaken until each seed became moistened with the solution. This was repeated several times, since the seeds tended to float together in a mass at the surface of the liquid. The period of exposure was fifteen minutes. Wilson transferred seeds from the sterilising solution to the culture medium without any rinsing in water. The writer found that the residue of the calcium hypochlorite interfered with the germination of the pine seeds in that the seeds started to swell, the testas split and the radicles could be seen, but they did not emerge. This difficulty was overcome by rinsing the seeds for two hours in sterile distilled water. Later it was noted that Fries and Forsman (1951) had observed that the length of time that seeds were rinsed appeared to be of some importance with regard to the subsequent exudation process. This is taken to mean that the rinsing affected the subsequent quantity and quality of the root exudates produced.

4. Fixing and Staining of Spores for Microscopic Examination.

Glass slides were washed with Chemico, dried

and polished. Haupt's Adhesive was dropped onto the slides and smeared to a thin film. Spores were shed from freshly harvested fruit bodies onto the slides and were left to dry over night. They were fixed in saturated mercuric chloride + 1% acetic acid for tweny four hours and washed in running water. Finally they were stained with cotton blue in lactophenol and mounted in Gurr's Neutral Mounting Medium.

Routine methods employed in Part 2 are the same as those described under "Materials and Methods" in Part 1.

The particular applications of special techniques to individual experiments are described under the appropriate experiments.

SECTION 2

Germination of Spores of B.luteus.

This investigation was undertaken in the clear knowledge that the germination of Boletus spores has, for a long time, been recognised as difficult and generally uncertain of attainment: Harley (1959), Fries (1941, 1943, 1949, 1966), Khudiakov and Vozniakovskaia (1951), Gehring (1957).

Because the original spores were collected late in the autumn of 1967, at the start of the research, it seemed advisable to take advantage of freshly harvested material to set up some experiments straight away, despite the fact that no preliminary reading had been undertaken. Gradually experimenting and reading together led to an accumulation of negative results which, in turn, pointed to the need to define one's terms of reference.

What is germination? For the purpose of this enquiry germination has been taken to be a process which leads to the first irreversible change in the

appearance of a spore. Manners (1966) states that this process takes place in three stages:-

1. Internal changes occurring within the confines of the spore wall.
2. The protrusion of the germ-tube from the spore wall.
3. The elongation of the germ-tube.

The spores of B.luteus are so dense that the internal changes could not be observed in the living spore. Thus it was necessary to rely on the two externally visible stages. In this respect, it was decided that if even a bulging of the spore could be induced, this would be taken as evidence that the germination process had started.

The first two experiments set up were based on observations made whilst collecting material in November 1967. Already there had been several nights of low temperatures, and, in consequence, there were numbers of frosted, decomposing sporophores to be found. The first experiment investigated the possibility that decaying fruit body material had an effect on the germination of spores. The second experiment was an attempt to find the effect of root

exudate from Pinus sylvestris on spore germination in Boletus, because the sporophores were always found in association with these trees.

B.luteus was the only species from which spores were harvested in 1967, and so it was used exclusively throughout this investigation. Unless otherwise stated, all the experiments were carried out in the dark in an incubator kept at a temperature of 18-20°C. The pH of culture media was kept to 5.5, because this was the pH of soil samples collected from Tentsmuir at the time of harvesting sporophores.

Experiment 1. Fruit Body Extract.

1 cm X 1 cm cubes of malt extract agar, (cut from strips as described in "Materials and Methods"), were placed on petri plates containing either malt extract agar or 2% glucose malt agar. 10 ml fruit body extract was added to each plate by dropping it from a sterilised pipette onto the agar cubes, to ensure direct contact between the spores and the fruit body extract. The extract was not added to

the control plates. The experiment was set up at fortnightly intervals from the middle of February till the beginning of April in 1968, 1969 and 1970. The spores had been harvested in November 1967, October 1968 and October 1969 respectively. The experiments were run for from eight to twenty two weeks and were only terminated when the plates dried out.

Every year many of the spore cubes showed signs of bacterial contamination. This led to the speculation that the presence of bacteria on the spores may have an effect on their germination.

In 1968 one out of four plates of 2% glucose malt agar + fruit body extract showed the development of mycelium which appeared to be characteristic of B.luteus, after five weeks. This success was not repeated in 1969 or 1970.

Two other media were tried in addition to the above in 1969 and 1970, namely sucrose malt agar and Czapek Dox (modified). No germination of spores was observed in the Czapek Dox plates, but in 1969

two out of two plates containing 3% sucrose malt agar + fruit body extract showed characteristic mycelium after three days, and two out of six plates showed the same development after six weeks.

Every time that mycelium which appeared to be characteristic of B.luteus was observed, subcultures were made for future examination. In no case did the mycelium develop any further, so the accuracy of the identification was not confirmed by microscopic examination.

Because of the bacterial activity on many of the spore cubes, the experiment was taken a stage further in 1970 to try to find out if the bacteria were present on the walls of the spores, or if they came from the fruit body extract. The same culture media were used but each plate was divided in half by the insertion of a barrier of sterilised asbestos cut to fit the internal diameter of the plate exactly. (Fig.4). No germination of spores was observed, but the development of bacteria both on the spore agar and on the fruit body extract was seen. At no time were visible colonies of bacteria seen to penetrate

the asbestos barrier.

Experiment 2. Root Exudates from Pine Seedlings.

In 1968 and 1969 the seeds of *Pinus sylvestris* harvested were found to be inviable. (See Materials and Methods). In 1970 difficulty was encountered in finding the right consistency of medium and combination of nutrients to keep the seedlings growing on a culture medium. The original intention was to find one which would keep the seedlings alive without exerting any undue influence on the *B.luteus* spores. Preliminary tests were conducted with water agar of 0.5%, 0.4% and 0.3% to find out which concentration could be penetrated by the radicles of the *P.sylvestris* seedlings. The 0.3% water agar was satisfactory, but the radicles did not penetrate the 0.5% or 0.4% media.

The experiment was set up in February and March of 1970. Nutrient broth set with 0.3% agar was poured into 250 ml flasks to a depth of approximately 1 cm. Four stratified and surface sterilised seeds of *P.*

sylvestris were added to each flask. Seven days after the emergence of the radicles had been observed, a 1 cm X 1 cm spore cube of B.luteus was added to each flask. As a control, one in four flasks had no P.sylvestris seeds. Half of the flasks were incubated at 18-20°C in the dark. The other half were kept in subdued daylight in the unheated laboratory at 12-17°C.

In most cases, the radicles of the seedlings failed to penetrate the agar. As its concentration was known to be satisfactory, the inference was that the culture medium was unacceptible to the seedlings. There was considerable elongation of the hypocotyl of the seedlings, but the cotyledons did not emerge completely from the testas, and became etiolated and less green with the passage of time. After four weeks a white fungus developed on some of the radicles. As it was not possible to identify it in St. Andrews, specimens were sent away with a request for identification. Dr.M.B.Ellis of the Commonwealth Mycological Institute, Kew, identified them as Cylindrocarpon destructans (Zins.) Schotten.

Matturi and Stenton (1964) say the primary habitat of Cylindrocarpon spp. is the root surface of plants. The saprophytic ability of this genus is low compared with that of vigorous soil saprophytes such as Fusarium culmorum and active colonisation of substrates is unlikely to occur readily. Inoculation experiments revealed at most a low degree of pathogenicity.

Experiment 3. Fresh Spores.

During the summers of 1968, 1969 and 1970 attempts were made to germinate freshly harvested spores. Within six hours of collecting sporophores, spores were shed onto petri plates containing malt extract agar or 2% glucose agar or 3% sucrose agar.

In 1968 and 1969 every plate was heavily contaminated, in less than twenty four hours, with species of Aspergillus and Penicillium. During this period, the same trouble was experienced when sub-culturing mycelium. The techniques employed were subjected to the closest scrutiny in an attempt to discover

the cause of the contamination. They were found to be above suspicion.

When the contamination was first experienced in 1968, an adjacent building was being subjected to extensive demolition and reconstruction. In 1969 a wall twelve yards down a corridor from the laboratory was broken open prior to the construction of a staircase and a lecture theatre. This common "building factor" led to the conclusion that unusual air disturbance had brought dust from the demolition and reconstruction and had led to the contamination. Support for this conclusion is given by the experience reported by Watling (1970 a).

The experiment was set up again throughout the summer of 1970, but no germination of spores resulted.

Experiment 4. (a) Orange Juice and
(b) Pollen Grains of P.sylvestris.

As a consequence of reading a paper by Chu Chou and Preece (1968), (a) fresh orange juice and (b) pollen

grains were tried as activators of germination in B.luteus spores.

The orange juice was used at two strengths, undiluted and diluted with sterilised distilled water to give a 20% solution. Agar cubes of spores were placed in petri plates and 10 ml orange juice was dropped onto each cube.

For the pollen grain investigation, staminate flowers of P.sylvestris were collected at Tentsmuir in June 1969, while they were shedding pollen. The flowers were carried to the laboratory in sterile McCartney bottles. There, they were shaken up vigorously to release the pollen grains and the flowers were removed from the bottles with flamed forceps. 10 ml sterilised distilled water was added to each bottle to dislodge the pollen grains which were sticking to the glass. This suspension was sterilised by autoclaving and was then added to spore cubes in petri plates in the manner described above. All the plates were incubated at 18-20°C in the dark.

Fourteen days later, all the plates were found

to be heavily contaminated in the manner described in Experiment 3.

Experiment 5. Soaking Spores in Distilled Water.

During the autumn of 1970 an attempt was made to look more closely at B.luteus spores for signs of germination. First of all freshly harvested spores, and later spores which had been stored in a refrigerator for four to twelve weeks at 10°C, were put into distilled water contained in a watch glass. With the aid of a water immersion lens they were kept under observation at intervals for twelve to fourteen days. At no time was any change noted in the size or shape of the freshly harvested spores. (Fig.5).

When spores which had been stored for six weeks were soaked in water for four hours, all increased in size and one in two hundred showed a terminal germ tube. (Fig.6).

The next two experiments were devised with a view to weakening the spore walls, the idea being that their inherent strength might be preventing any necessary swelling of the spores or the emergence of the germ tubes. It was also hoped that, if any blockage of the germ pore could be removed, its position in the spore might be ascertained.

Experiment 6. Soaking Spores in Enzyme Solution.

Cocking (1960) showed that cellulase could be used as a means of releasing protoplasts from plant cells. Two sources of cellulase were used, namely

Cellulase Type 1 from *Aspergillus niger*

Cellulase Type 111 from *Rhizopus* mould.

Both were supplied by Sigma London Chemical Co. Ltd., and were stored at -10°C. The cellulase was activated by the method of Bromwich (1970).

10 g sucrose were dissolved in 50 ml 0.02M phosphate buffer (pH 6.0). 0.25 g cellulase was dissolved in this solution, now referred to as the Enzyme Solution. The pH was corrected to 5.4

using 2N HCl.

A measured quantity (10 ml) of Enzyme Solution was poured into a glass dish and spores, which had been cut from their agar strip, were stirred in. After thirty minutes a further 10 ml Enzyme Solution was added. The dish was covered with a glass plate and was allowed to stand for three hours at room temperature (21°C).

The first time this experiment was set up, freshly harvested spores were used. The second time spores which had been stored at 10°C for twenty two weeks were used. In the former case, two spores out of approximately one hundred and fifty showed a marked bulge on one side after four hours. In the latter case, two spores out of approximately two hundred showed a change of shape, again after four hours. One bulged laterally and the other appeared to show a terminal germ tube. (Fig.7). The spores had all doubled in size.

This experiment provided evidence that soaking spores in a cellulase enzyme solution increases the

size of the spores but does not increase the rate of germination. It showed that some spores of B.luteus remain viable after nearly five months storage. It also suggested that germination may sometimes be by means of a lateral germ pore, a fact shown in Plate 5 of a paper by Fries (1941).

Experiment 7. Soaking Spores in Sodium Hypochlorite Solution.

Again an attempt was made to soften the spore walls, this time using sodium hypochlorite (Lowry and Sussman, 1958). Spores of B.luteus still adhering to their agar strip were placed in a 1% solution of sodium hypochlorite contained in a watch glass. The mixture was allowed to stand at room temperature for sixty minutes. Thereafter the spores were rinsed repeatedly with sterile distilled water, until the smell of sodium hypochlorite could no longer be detected.

The most noticable effect of this treatment was a slight increase in the overall size of the spores. (Fig.8). One out of about one hundred and fifty

spores produced what appeared to be a terminal germ tube.

Experiment 8. Simultaneous Repeat of Earlier Experiments.

The object of this experiment was to eliminate any variations which might be caused by setting up experiments at different dates or with spores of different ages.

The experiment was set up on 13.2.71 using spores harvested on 10.10.70. It was allowed to run for nine weeks.

The treatments were as follows:-

(a) Spores soaked in sterile distilled water;

(b) Spores soaked in sterile distilled water + fruit body extract;

(c) Spores soaked in 2% glucose solution;

(d) Spores soaked in 2% glucose solution + fruit body extract;

(e) Spores soaked in 3% sucrose solution;

(f) Spores soaked in 3% sucrose solution + fruit

body extract;

(g) Spores on 2% glucose malt agar;

(h) Spores on 2% glucose malt agar + fruit body extract;

(i) Spores on 3% sucrose malt agar;

(j) Spores on 3% sucrose malt agar + fruit body extract;

(k) Spores on malt extract agar;

(l) Spores on malt extract agar + fruit body extract;

(m) Spores on sand + nutrient solution;

(n) Spores on sand + nutrient solution + pine seeds;

(o) Spores on nutrient agar;

(p) Spores on nutrient agar + pine seeds.

Treatments (a)-(f) each comprised four replicates. The solutions were contained in watch glasses standing in petri plates lined with damp filter paper.

Treatments (g)-(l) each comprised four replicates and were set up in the manner outlined in Experiment 1.

Treatments (m)-(p) again comprised four replicates each. For Treatments (m) and (n) 100 g of sterilised Tay River sand was put into each of eight 250 ml flasks and 25 ml of nutrient solution was added. This was the solution used by Agnihotri and Vaartja (1967) for

collecting root exudate from red pine seedlings, (Appendices). Spores were added to all the flasks, but stratified, surface sterilised seeds of P.sylvestris were added to only four flasks (10 seeds/flask).

Treatments (o) and (p) varied from Treatments (m) and (n) in that the nutrient solution was set with 0.5% agar, instead of being added to sand. (At lower concentrations than 0.5% agar, the nutrient medium would not set). The depth of the agar was approximately 1 cm. Treatments (m),(n),(o) and (p) were kept in a growth chamber with eighteen hours daylight, a temperature of 21°C and no humidity, apart from that provided by the nutrient medium.

Results.

Treatments (a)-(f) inclusive.

No sign of germination was observed. Spores were examined under the microscope. There was no change in their overall shape, but the ones in distilled water had increased in size.

Treatments (g)-(l) inclusive.

After five weeks and two days, all four plates of 2% glucose malt agar without fruit body extract showed development of hyphae which appeared to be characteristic of B.luteus. One plate of malt extract agar without fruit body extract showed development of similar hyphae after nine weeks. None of the other plates showed any sign of germination.

Treatments (m) and (n).

Four days after the experiment was set up, six to eight seeds per flask germinated and grew rapidly. Three weeks after germinating, the hypocotyls of the seedlings turned red and the radicles turned black and broke off. As there was no pathogen present, it is suggested that the sand had been too sharp and had amputated the seedlings.

Treatments (o) and (p).

Again, six to eight seeds per flask germinated within four days of setting up the experiment. The seedlings grew vigorously. After seven weeks "short roots" (Harley, 1959) were seen to be developing. This is a point in favour of using a clear medium such as agar rather than sand.

In Treatments (m)-(p) inclusive there were no signs of germination of spores.

Discussion.

Over the years that the writer has been considering this problem, it seems that any one of three possibilities may explain why it is so difficult to germinate spores of B.luteus.

It may be that (a) the spores require a combination of environmental factors which are not easily supplied under laboratory conditions; or (b) they may pass through a period of dormancy which is hard

to break; or it may be that (c) there is very poor germination of spores because Boletus has reached a stage of evolution at which it is no longer dependent on the germination of spores to extend the range of the genus. As Gregory (1952) suggests with other Basidiomycetes, it may be that a few spores germinate merely in order to fuse with an already established mycelium, thus ensuring the dispersal of genes.

The recorded work on the germination of Boletus shows that the problem has been tackled from a wide variety of angles.

Melin (1925, 1962) made use of unidentified stimulating substances found in the secretions of germinating pine seeds. These unknown substances, known as the M-factor, conspicuously affected the germination of basidiospores of Basidiomycetes. This did not happen in the writer's experiments.

Fries (1941, 1943, 1948) investigated the use of yeasts as activators of germination. He also explored the effects of a synthetic nutrient medium; irradiation with ultra violet light; treating with

pepsin; drying and moistening alternately.

Gehring (1957) approached the problem by examining the effects of vitamin B-complex; chitinbacteria; and other stimuli such as nicotinamide, ascorbic acid, glutathione, extracts of fructifications and soils; anaerobic medium and temperature stimuli.

Khudiakov and Vozniakovskaia (1951) tried the effect of synthetic culture media.

All these workers record the achievement of a germination rate of about 1%, but there is no indication that they succeeded in accelerating the rate or increasing the percentage of germination.

The low percentage of germination achieved by workers using synthetic nutrient media led the writer to the use of other things. The first experiment set up and repeated annually for four years investigated the stimulating effect of fruit body extract. As noted by Fries (1943) and Gehring (1957) germination did occur. Although the fruit body extract was a constant factor in all four years, the germin-

ation occurred in plates containing a 2% glucose malt extract agar in 1968; in plates containing a 3% sucrose malt agar in 1969; in no plates in 1970 and in plates containing a 2% glucose malt extract agar in 1971. This pattern suggests that the presence of sugar is necessary for the germination process to be initiated. Hawker (1966) notes that spores which normally produce germ-tubes only after considerable swelling are unable to germinate in the absence of glucose. In 1971 the writer soaked spores in sterile distilled water, 2% glucose solution and 3% sucrose solution. Some of the spores in distilled water increased in size, but there was no measurable change in the spores soaking in the sugar solutions. These results are not in agreement with the findings of Hawker.

Experiment 8 (m),(n),(o),(p) was also designed to test the effect of stimulating substances, namely exudates from the roots of pine seedlings. After ten weeks, no germination of spores had occurred. Melin (1962) germinated spores of B.luteus in a "maximum" nutrient medium containing sugar and salts, ten B-vitamins and a mixture of nineteen amino acids.

Germination took four weeks, but the addition of excised roots of pine seedlings reduced the time to fourteen days. If the work is being continued, this is an experiment which ought to be repeated, as the results sound so promising.

As far as the writer could discover, there are no records of the enzyme cellulase being used to initiate germination, although Fries (1948) made unsuccessful attempts to use pepsin for this purpose. Experiment 6 supplies evidence that germination began, but not that it was stimulated by cellulase because the rate of less than 1% is so low.

Lowry and Sussman(1958) treated ascospores of Neurospora tetrasperma with sodium hypochlorite in order to study their surface. With the assistance of this substance they were able to establish the presence of three layers in the cell walls. The ascospores swelled sufficiently to reveal the position of the germ pores. Experiment 7 was set up to see if sodium hypochlorite would reveal any details concerning the spore wall of B.luteus. Although an increase in spore size was noted, the

appearance of the spore wall was not altered as seen under the light microscope.

Concerning the existence of a dormant period, the writer had no success with germinating freshly harvested spores, and had most success with spores which had been stored at 10°C for four to five months. Fries (1943) could not establish that the spores of soil inhabiting Hymenomycetes need a certain, relatively long period of rest to be able to germinate. But he did establish the following facts in connection with the viability of spores of species of Boletus:-

(a) spores from different fruit bodies of the same species have varying capacity of germinating;

(b) in different collections of spores of the same species, the capacity to germinate diminishes with varying rapidity;

(c) B.luteus seems to retain viability longer than most;

(d) storing of spores at low temperature (-10°C) prolongs the time during which they are viable.

Repeatedly in the literature one reads that the basidiospores of coprophilous and lignicolous Basidio-

mycetes germinate readily and rapidly. As Fries (1966) points out, these fungi live on relatively ephemeral substrates which may reflect their need for a highly efficient dispersal mechanism.

On the other hand, mycorrhiza-forming fungi such as species of Boletus, which persist in a given area for a considerable number of years do not have the same urgency to reproduce themselves from their basidiospores. This in turn means that they are not handicapped by producing fewer viable spores with a slower rate of germination. Hence the difficulty in germinating them speedily and in quantity under laboratory conditions. The production of fewer viable spores and the slower rate of germination do not, however, lead to the failure of the species to maintain themselves.

A P P E N D I X A

Formulae for Culture Media and Buffer Solution.

Malt Extract Agar

Oxoid No. 1 Agar	6 g
Malt Extract	3.5 g
Distilled Water	400 ml

2% Glucose Malt Agar

Oxoid No. 1 Agar	6 g
Glucose	8 g
Malt Extract	14 g
Distilled Water	400 ml

3% Sucrose Malt Agar

Oxoid No. 1 Agar	6 g
Sucrose	12 g
Malt Extract	14 g
Distilled Water	400 ml

Czapek Dox Agar (modified)

Add 45.4 g to 1 litre of distilled water and soak for 15 minutes. Sterilise by autoclaving for 20 minutes at 10 lb per square inch. Mix well before pouring.

Formula per litre

Sodium nitrate	2.0 g
Potassium chloride	0.5 g
Magnesium glycerophosphate	0.5 g
Ferrous sulphate	0.01 g
Potassium sulphate	0.35 g
Sucrose	30.0 g
Oxoid Agar No. 3	12.0 g

Nutrient Broth

Dissolve 13 g in 1 litre distilled water. Mix well, distribute into final containers and sterilise.

Formula per litre

'Lab-Lemco' Beef Extract	1 g	
Yeast Extract (Oxoid L 20)	2 g	
Peptone (Oxoid L 37)	5 g	
Sodium chloride	5 g	pH 7.4 (approx.)

Water Agar or Nutrient Broth Agar

To a flask containing 100 ml liquid, add agar at the rate of 0.5 g, 0.4 g or 0.3 g to make a o.5%, 0.4% or 0.3% medium.

Nutrient Solution of Agnihotri and Vaartaja (1967)

KH_2PO_4	300 mg
$Ca(NO_3)_2.4H_2O$	100 mg
$NaNo_3$	300 mg
$MgSO_4.7H_2O$	100 mg
NaCl	25 mg
$FeCl_3.6H_2O$	1mg
Distilled water	1000 ml

Peptone Dextrose Agar plus rose bengal and streptomycin

(Martin 1950)

Agar	20.0 g
KH_2PO_4	1.0 g
$MgSO_4.7H_2O$	0.5 g
Peptone	5.0g
Dextrose	10.0 g
Distilled water	1000 ml

Rose bengal 1:30,000 Streptomycin 30 μg/ml

Preparation: All the materials except rose bengal and antibiotic are dissolved in water. The mixture is heated slowly while stirring until it starts to boil. It is removed from the heat and a 1:300 dilution of rose bengal is added at the rate of 1 ml/100 ml of medium. After bottling and autoclaving and before pouring plates, streptomycin (made up in 50% alcohol) is added to the cooled liquid medium.

Ferry and Das Medium (1968)

Glucose	20 g		
NH_4Cl	0.5 g	Malt extract	5.0 g
$MgSO_4.7H_2O$	0.5 g	Oxoid agar	15 g
KH_2PO_4	0.5 g		

Basal Medium of Lilly and Barnett (1951)

Carbon source (glucose) 10 g

Nitrogen source ≐ 2 g asparagine

KH_2PO_4	1 g		$MgSO_4.7H_2O$	0.5 g	
Fe+++	0.2 mg,	Zn++	0.2 mg,	Mn++	0.1 mg
Biotin	5 µg		Thiamine	100 µg	

Distilled water to make 1 litre

Selective Medium for the Isolation of Basidiomycetes

(Russell, 1956)

Oxoid desiccated malt extract	3 g
Oxoid mycological peptone	0.5 g
o-Phenyl phenol	0.006 g
Distilled water	100 ml

The medium is autoclaved at 10 lb/sq. inch for 10 minutes. The required amount of o-phenyl phenol is added from a stock solution prepared by dissolving 1 g in 50 ml industrial alcohol. This solution is diluted to 100 ml with distilled water.

Preparation of Phosphate Buffer

(Dawson, Elliott, Elliott and Jones, 1959)

Weigh out 17.805 g $Na_2HPO_4.2H_2O$, put into 250 ml water and add 250 ml water. Solution A.

Weigh out 78.525 g $NaH_2PO_4.2H_2O$, put into 250 ml water and add 250 ml water. Solution B.

For pH 6.0 take:

(12.3 x 2.5) ml A and (87.7 x 2.5) ml B

Mix together and make up to 500 ml.

Check pH = 6.0

A P P E N D I X B

Table 1.

Nitrogen Sources		Wt. in gm/litre
Potassium nitrate	KNO_3	2.69
Potassium nitrite	KNO_2	2.27
Ammonium tartrate	$(NH_4)_2C_4H_4O_6$	2.45
dl Alanine	$CH_3.CH(NH_2)COOH$	2.37
dl Asparagine	$NH_2CO.CH_2CH(NH_2).COOH$	2.00
Glycine	$NH_2.CH_2COOH$	2.00
dl Methionine	$CH_2(SCH_3)CH_2.CH(NH_2).COOH$	3.97

Lilly and Barnett (1951)

Table 2 Dutch Cultures, 1970.

The effect of the source of nitrogen on the growth of 5 species of Boletus. Incubation period 4 weeks at 22°C. Dried to constant weight at 50°C. Average of 2 replicates in mg. Initial pH = 6.0

Nitrogen Source	B. badius	B. bovinus	B. edulis	B. luteus	B. variegatus
Control (-N)	52.45	43.45	89.65	78.20	60.15
filtrate pH	6.3	5.8	5.75	5.6	6.2
Pot.nitrate	155.4	65.9	132.7	96.85	74.3
filtrate pH	7.1	6.3	8.9	6.3	6.2
Pot.nitrite	100.65	99.8	149.35	88.0	76.9
filtrate pH	6.3	6.2	8.8	6.2	6.1
Amm.tartrate	137.75	102.2	163.2	60.75	66.7
filtrate pH	3.1	4.8	3.2	4.7	4.5
Alanine	168.5	76.65	130.4	66.3	84.6
filtrate pH	3.8	5.5	7.4	4.9	4.0
Asparagine	192.35	91.4	123.75	77.85	58.4
filtrate pH	3.9	4.8	7.7	4.6	3.6
Glycine	195.3	133.25	149.85	81.9	73.15
filtrate pH	6.8	4.6	7.6	4.9	4.4
Methionine	99.3	114.65	123.35	80.0	73.15
filtrate pH	4.3	4.7	3.4	4.8	4.1

Table 3 Tentsmuir Cultures, 1971.

The effect of the source of nitrogen on the growth of 2 species of *Boletus*. Incubation period 4 weeks at 22°C. Dried to constant weight at 50°C. Average of 2 replicates, in mg. Initial pH = 6.0

Nitrogen Source	*B.badius*	*B.variegatus*
Control (-N)	52.45	19.55
filtrate pH	6.3	4.55
Pot. nitrate	154.9	118.75
filtrate pH	7.1	7.9
Pot. nitrite	104.7	61.55
filtrate pH	6.3	6.1
Amm. tartrate	141.15	131.75
filtrate pH	3.1	4.7
Alanine	165.95	135.35
filtrate pH	3.8	6.5
Asparagine	199.15	113.95
filtrate pH	3.9	7.0
Glycine	195.95	145.4
filtrate pH	6.8	6.4
Methionine	100.1	34.3
filtrate pH	4.3	4.3

Table 4 Dutch Cultures, 1970.

The effect of the source of carbon on the growth of 5 species of *Boletus*. Incubation period 4 weeks at 22°C. Dried to constant weight at 50°C. Average of 2 replicates in mg. Initial pH = 6.0

Carbon Source	*B. badius*	*B. bovinus*	*B. edulis*	*B. luteus*	*B. variegatus*
Control (no +C)	19.65	10.95	20.0	9.60	1.85
filtrate pH	7.8	7.8	8.4	7.0	6.9
Glucose	160.3	91.7	160.3	56.8	83.8
filtrate pH	3.6	5.3	6.0	5.5	5.9
Sucrose	145.6	173.15	163.7	87.0	91.6
filtrate pH	7.0	6.15	5.2	6.2	6.1
Maltose	123.3	73.2	128.0	92.1	63.8
filtrate pH	4.0	5.7	4.4	6.6	6.2
Fructose	143.85	46.4	125.4	67.4	84.1
filtrate pH	4.1	6.0	4.5	6.2	6.2
Starch	211.6	378.3	157.1	285.8	350.75
filtrate pH	4.2	6.5	7.5	6.1	7.3

Table 5 Tentsmuir Cultures, 1971.

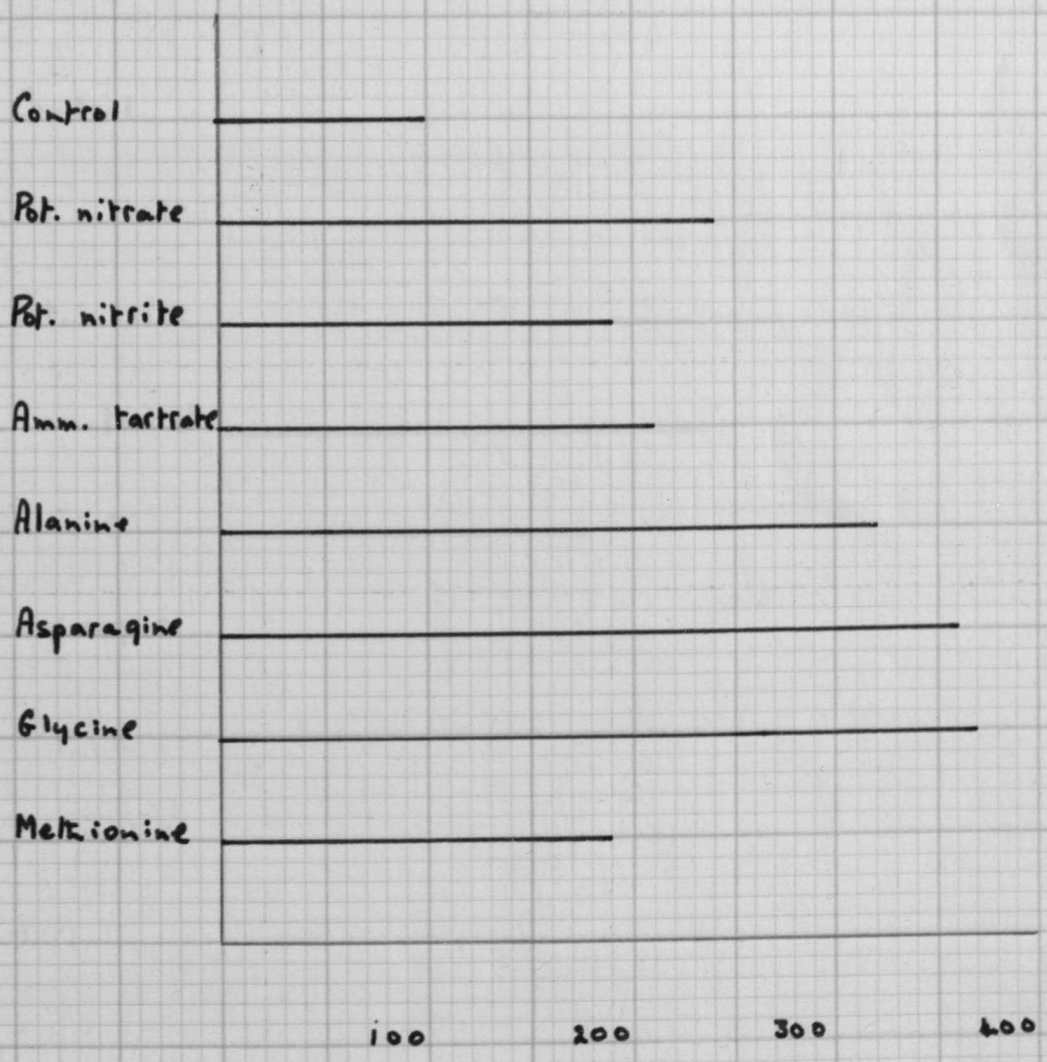
The effect of the source of carbon on the growth of 2 species of *Boletus*. Incubation period 4 weeks at 22°C. Dried to constant weight at 50°C. Average of 2 replicates in mg. Initial pH = 6.0

Carbon Source	*B.badius*	*B.variegatus*
Control (no +C)	19.7	76.7
filtrate pH	7.8	7.9
Glucose	159.05	119.3
filtrate pH	3.6	7.6
Sucrose	145.3	176.1
filtrate pH	7.0	7.6
Maltose	123.65	191.9
filtrate pH	4.0	6.8
Fructose	144.3	140.4
filtrate pH	4.1	7.8
Starch	191.65	174.05
filtrate pH	4.2	7.0

APPENDIX C

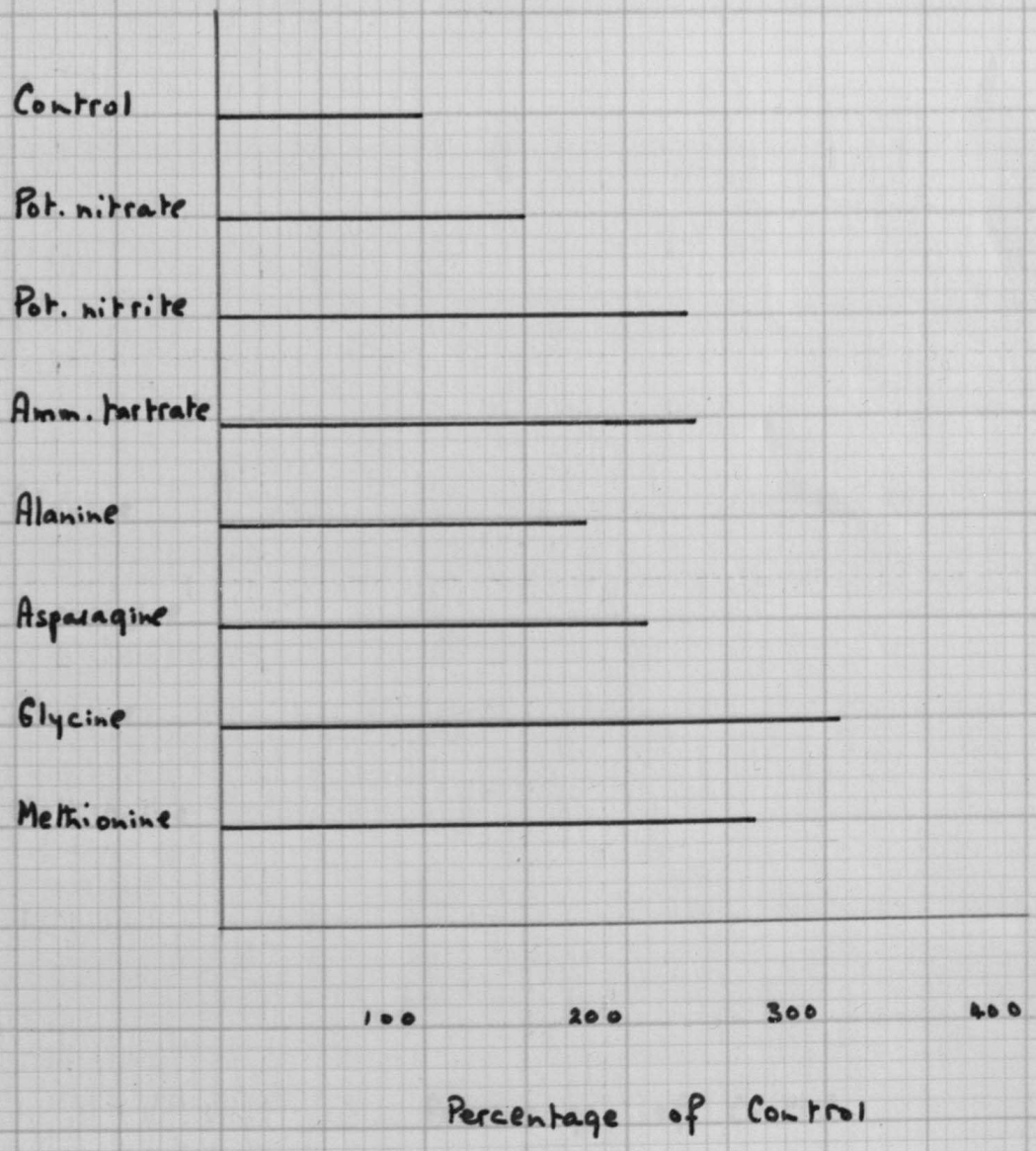
Nitrogen Utilisation

<u>Boletus badius</u> (Dutch)



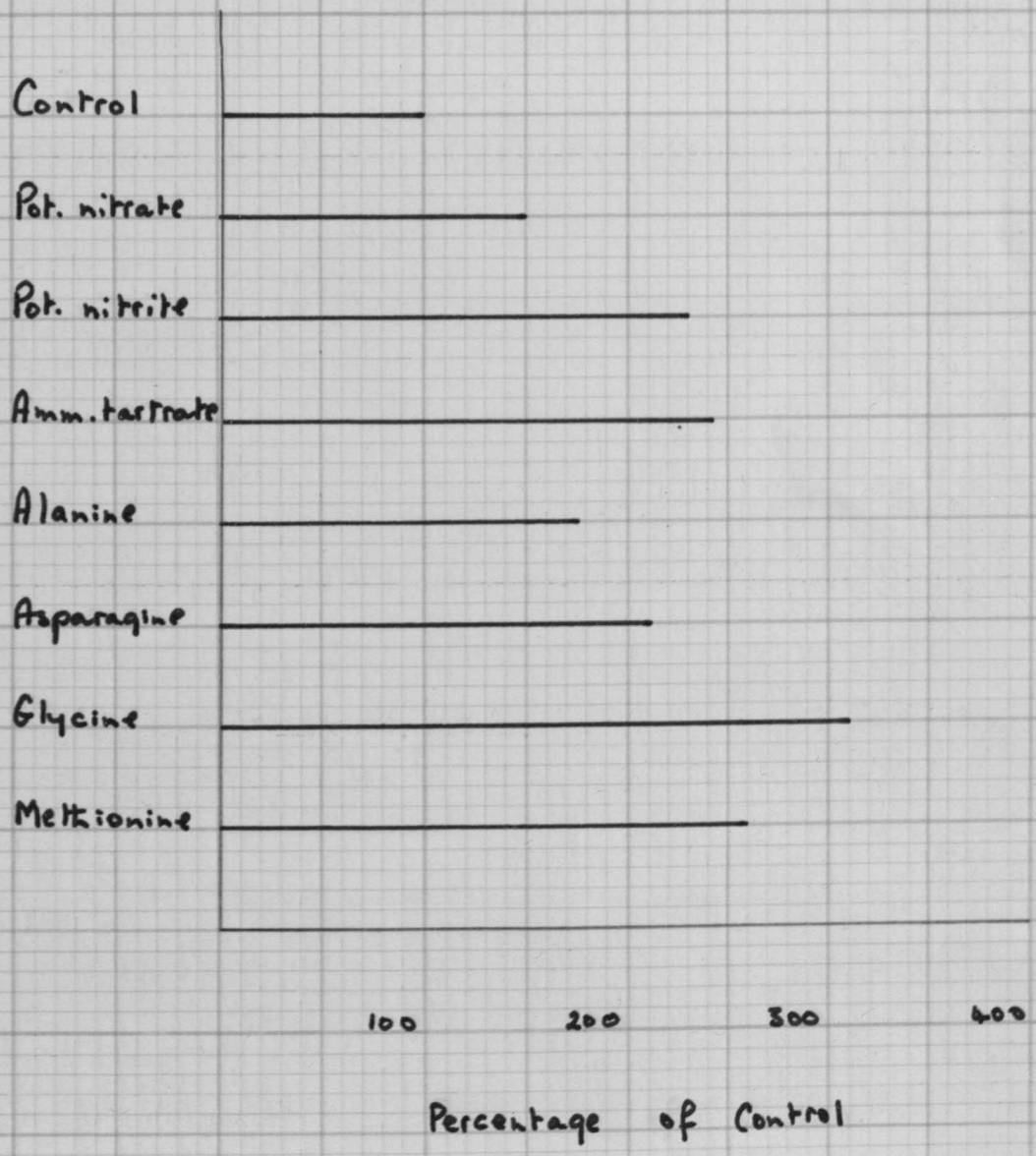
Nitrogen Utilisation

Boletus bovinus (Dutch)



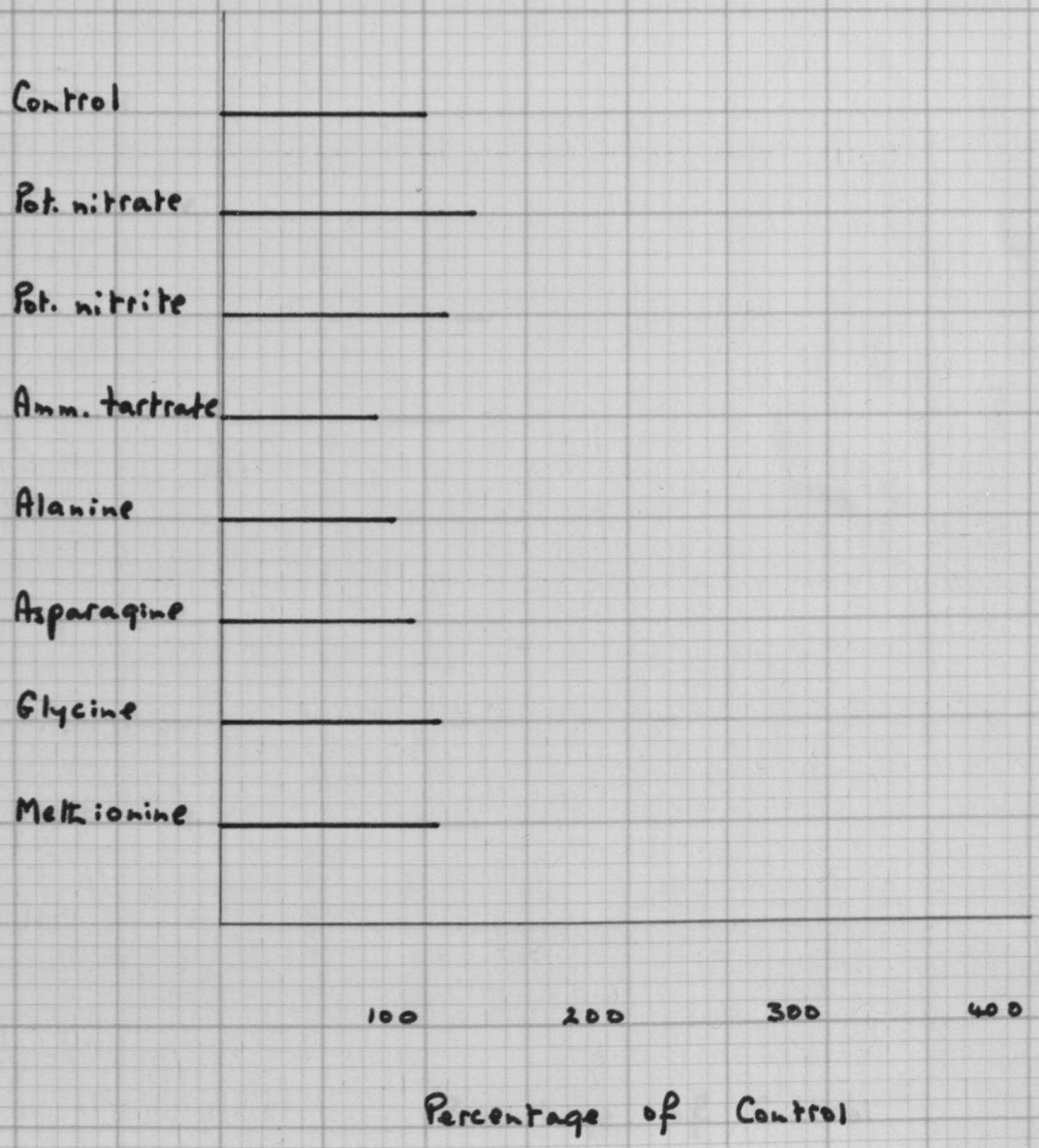
Nitrogen Utilisation

Boletus edulis (Dutch)



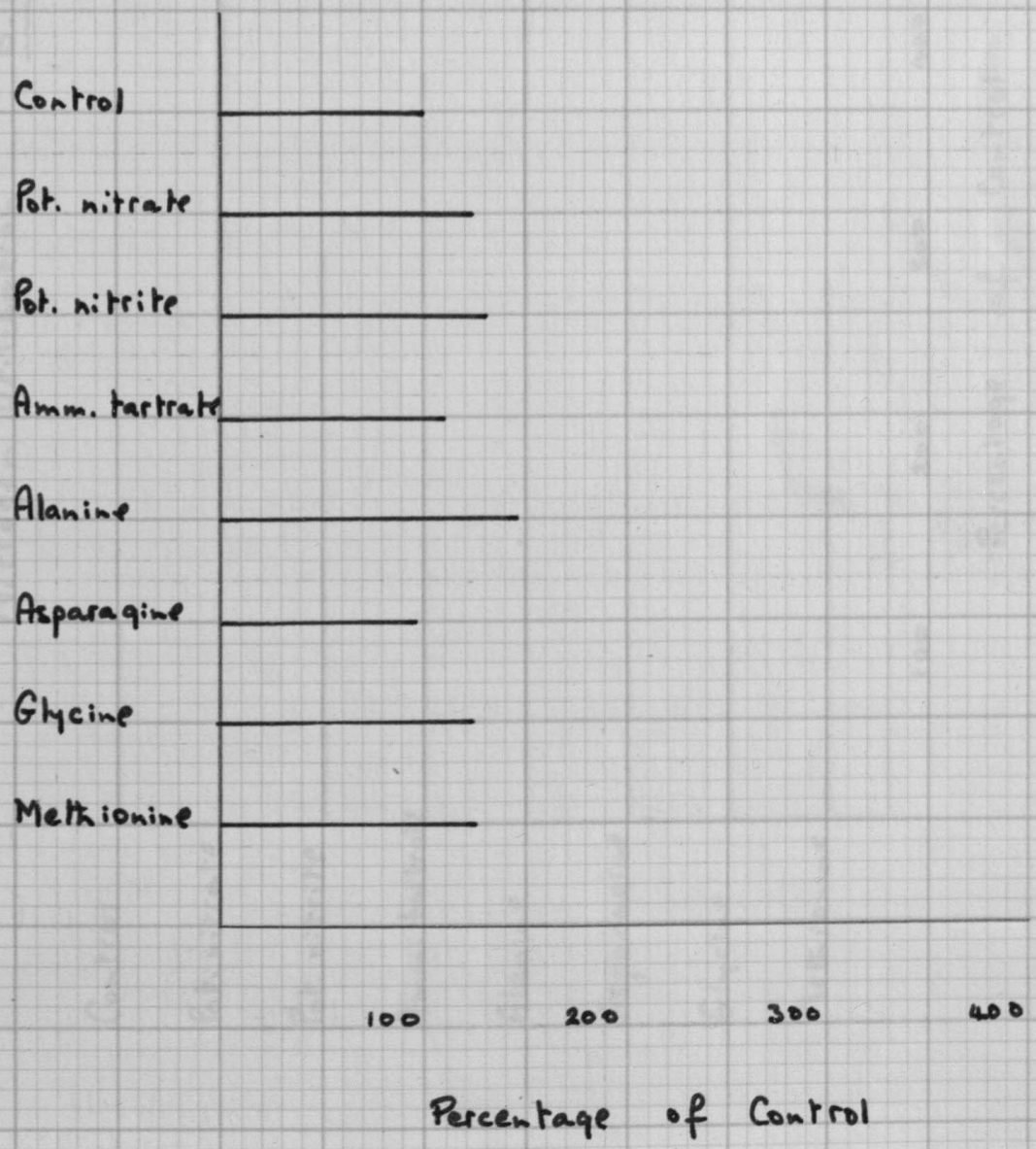
Nitrogen Utilisation

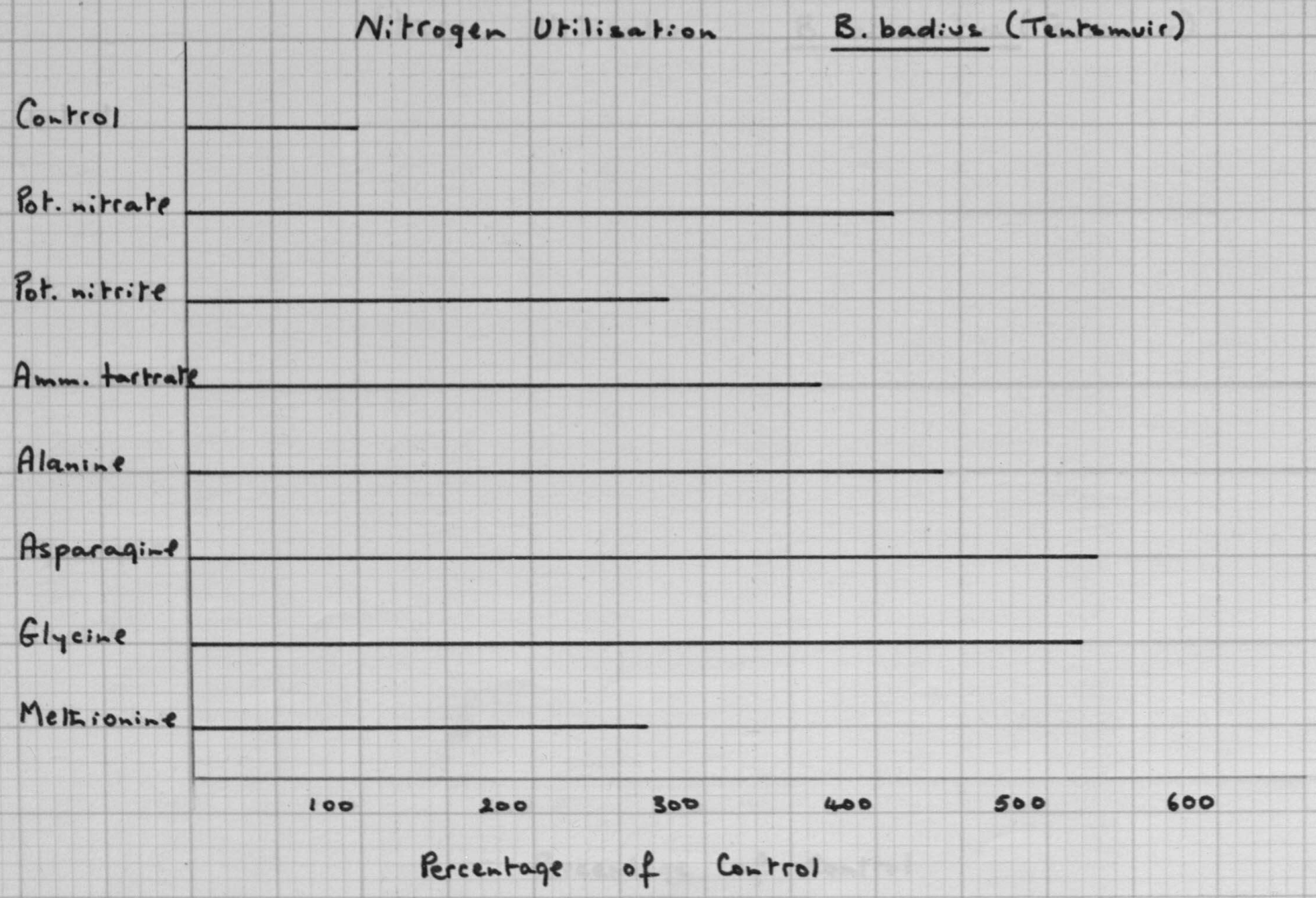
Boletus luteus (Dutch)

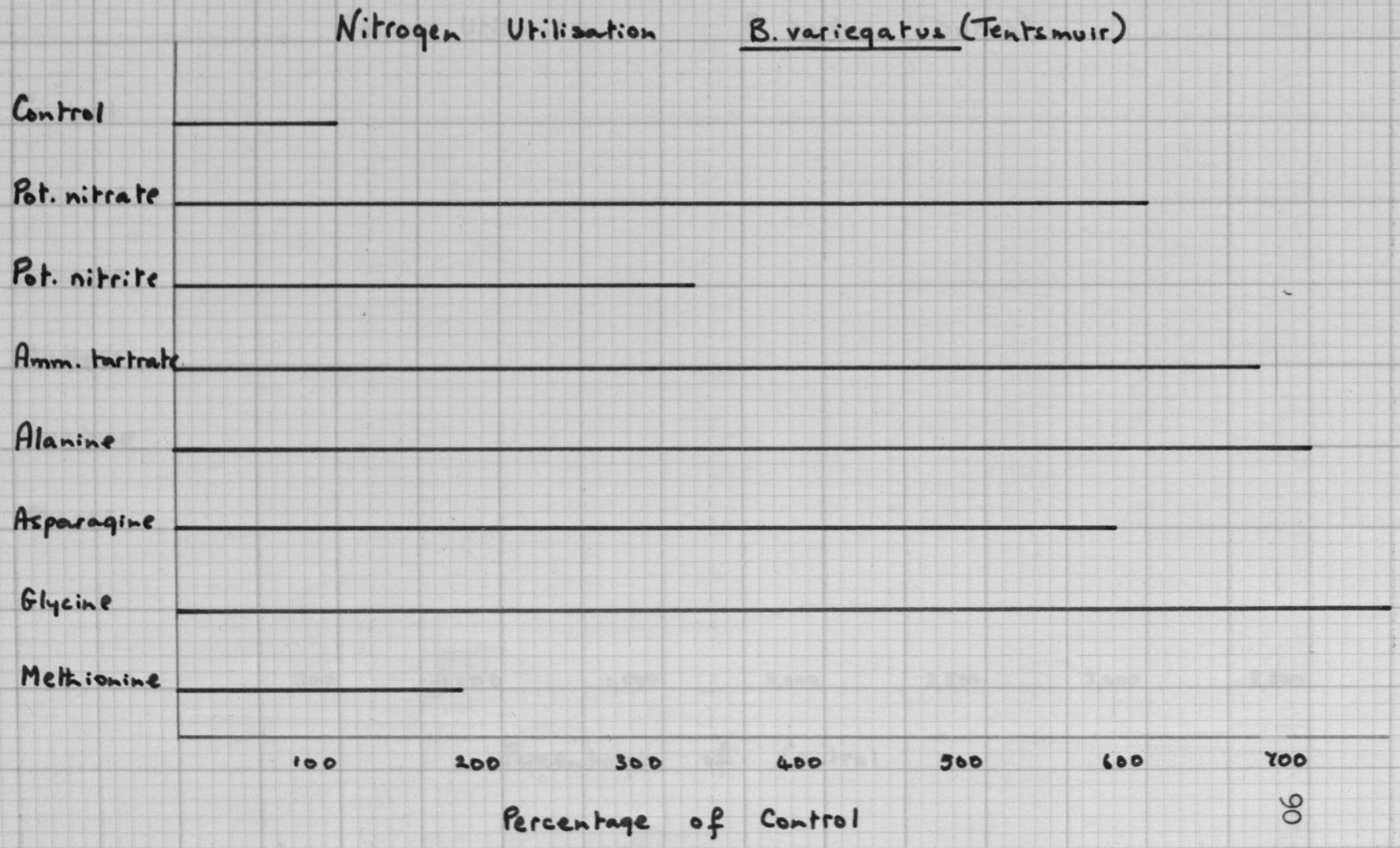


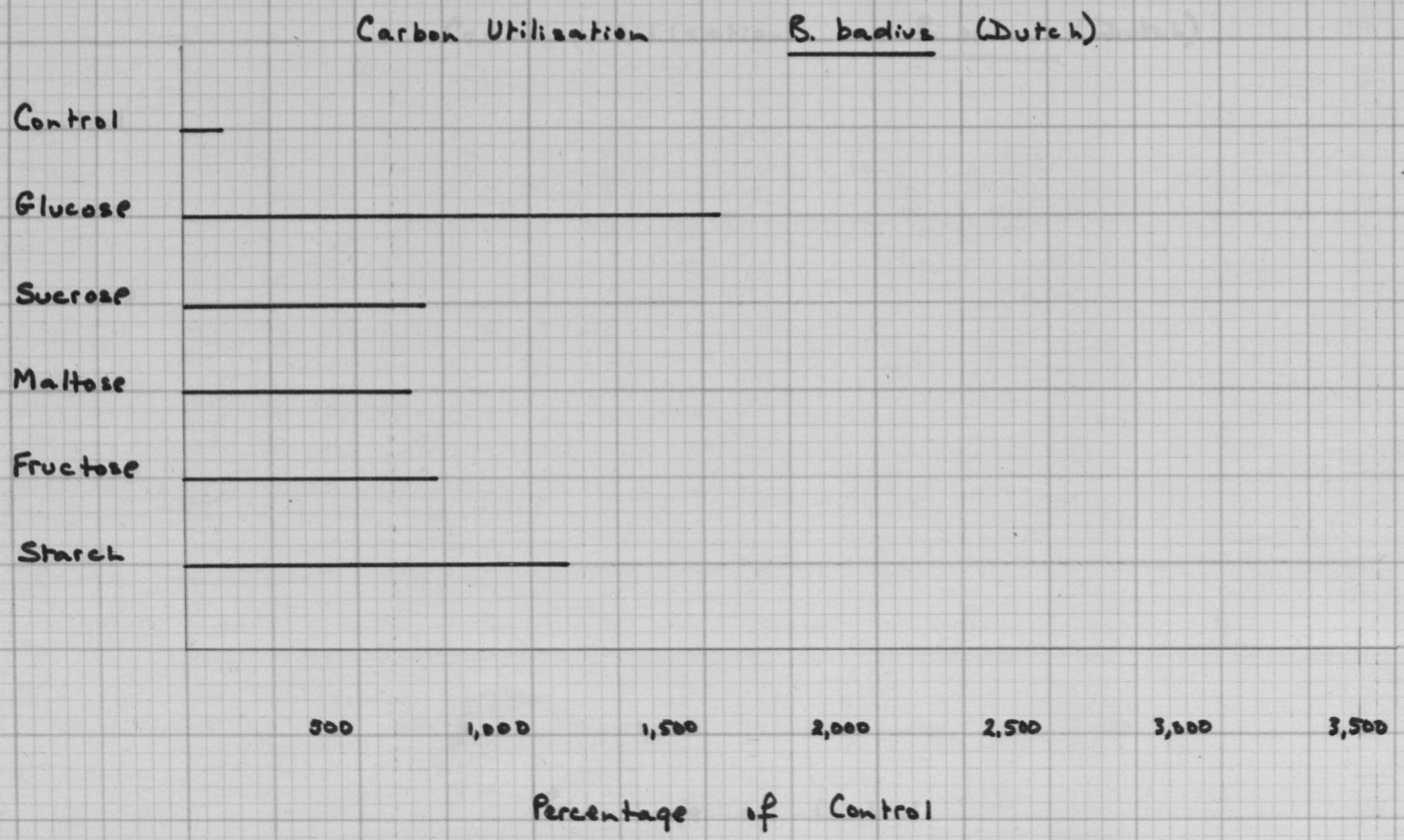
Nitrogen Utilisation

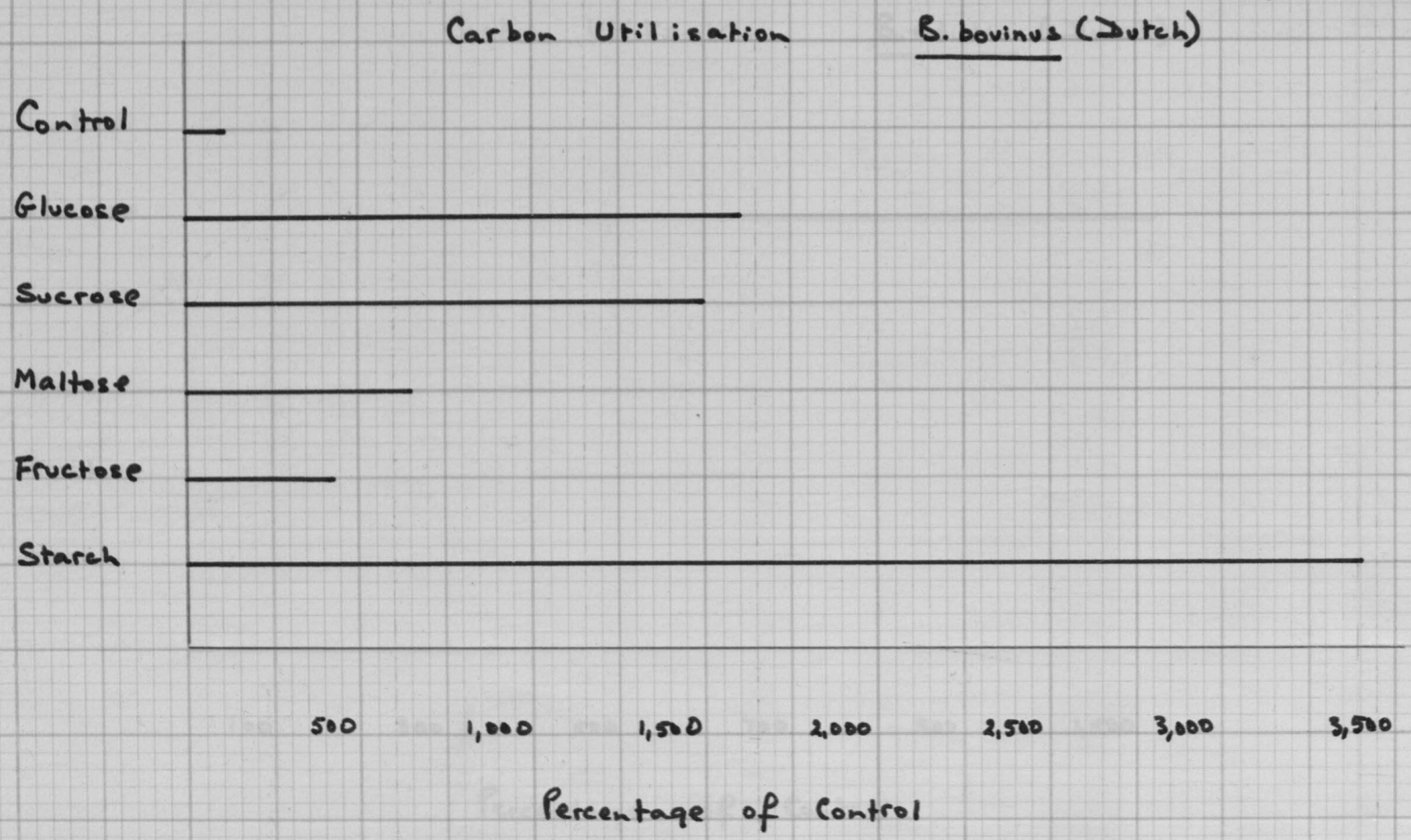
Boletus variegatus (Dutch)

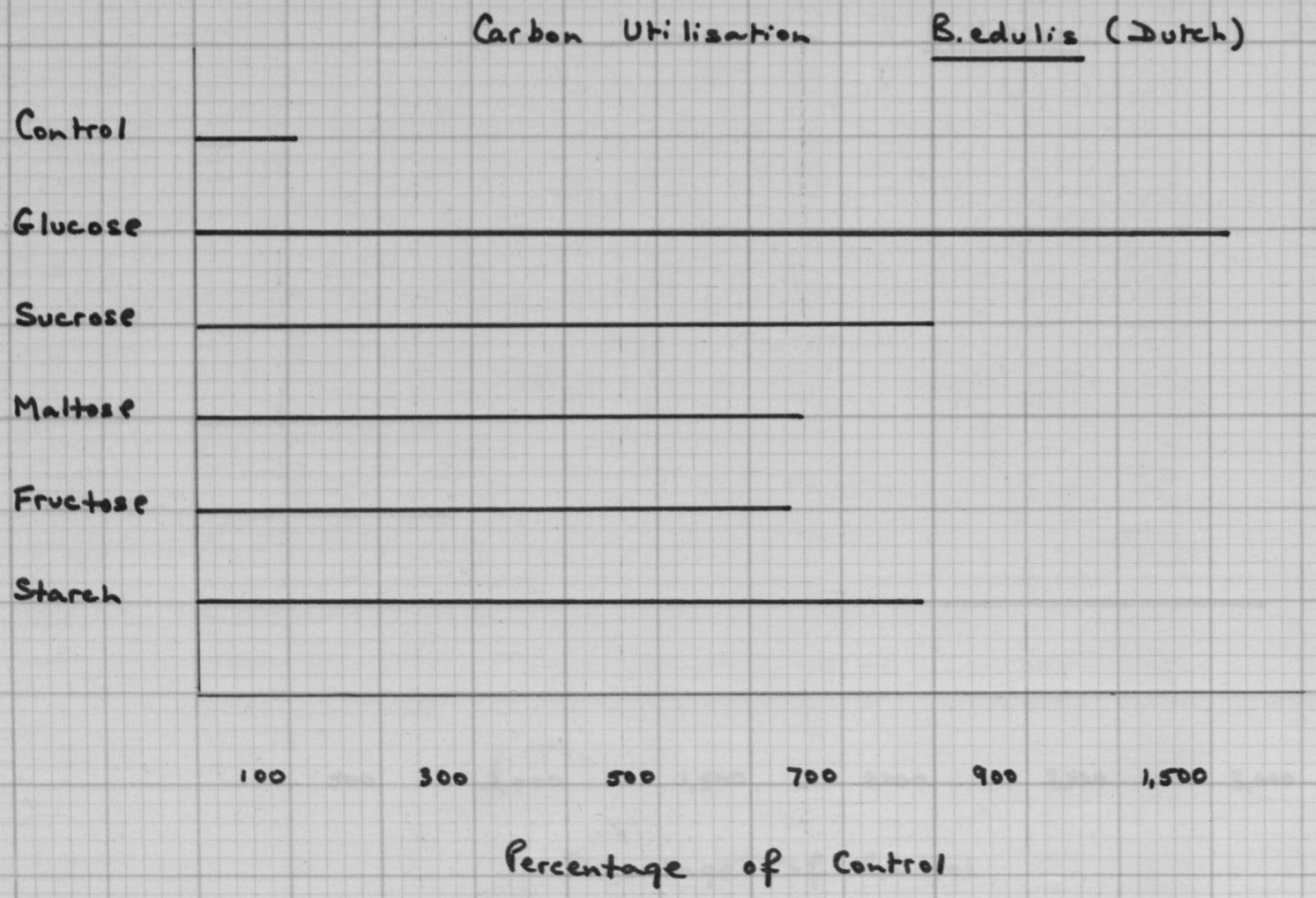



Nitrogen Utilisation
B. badius (Tentamuir)
Control
Pot. nitrate
Pot. nitrite
Amm. tartrate
Alanine
Asparagine
Glycine
Methionine
100
200
300
400
500
600
Percentage of Control

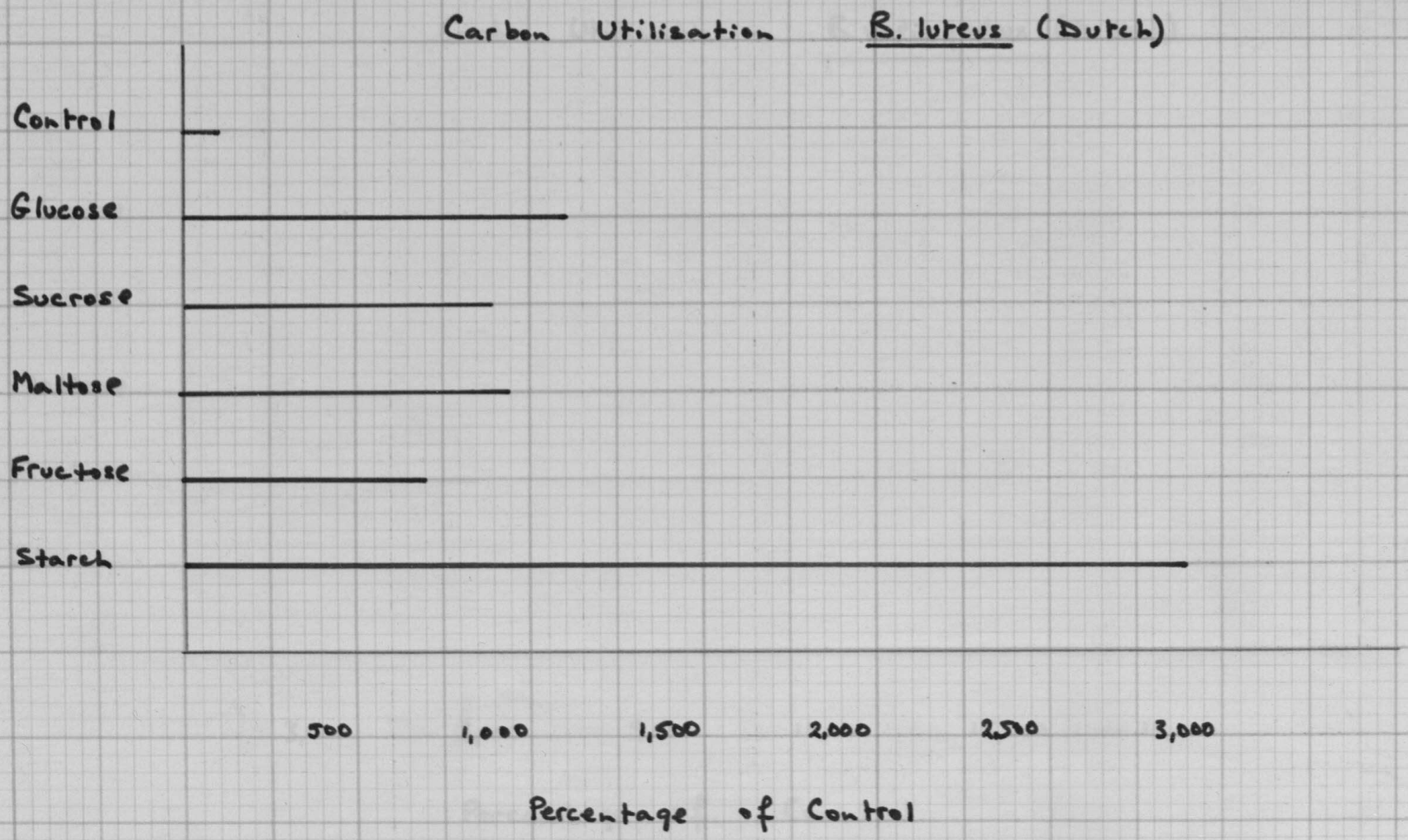

Nitrogen Utilisation
B. variegatus (Tentsmuir)
Control
Pot. nitrate
Pot. nitrite
Amm. tartrate
Alanine
Asparagine
Glycine
Methionine
100
200
300
400
500
600
700
Percentage of Control

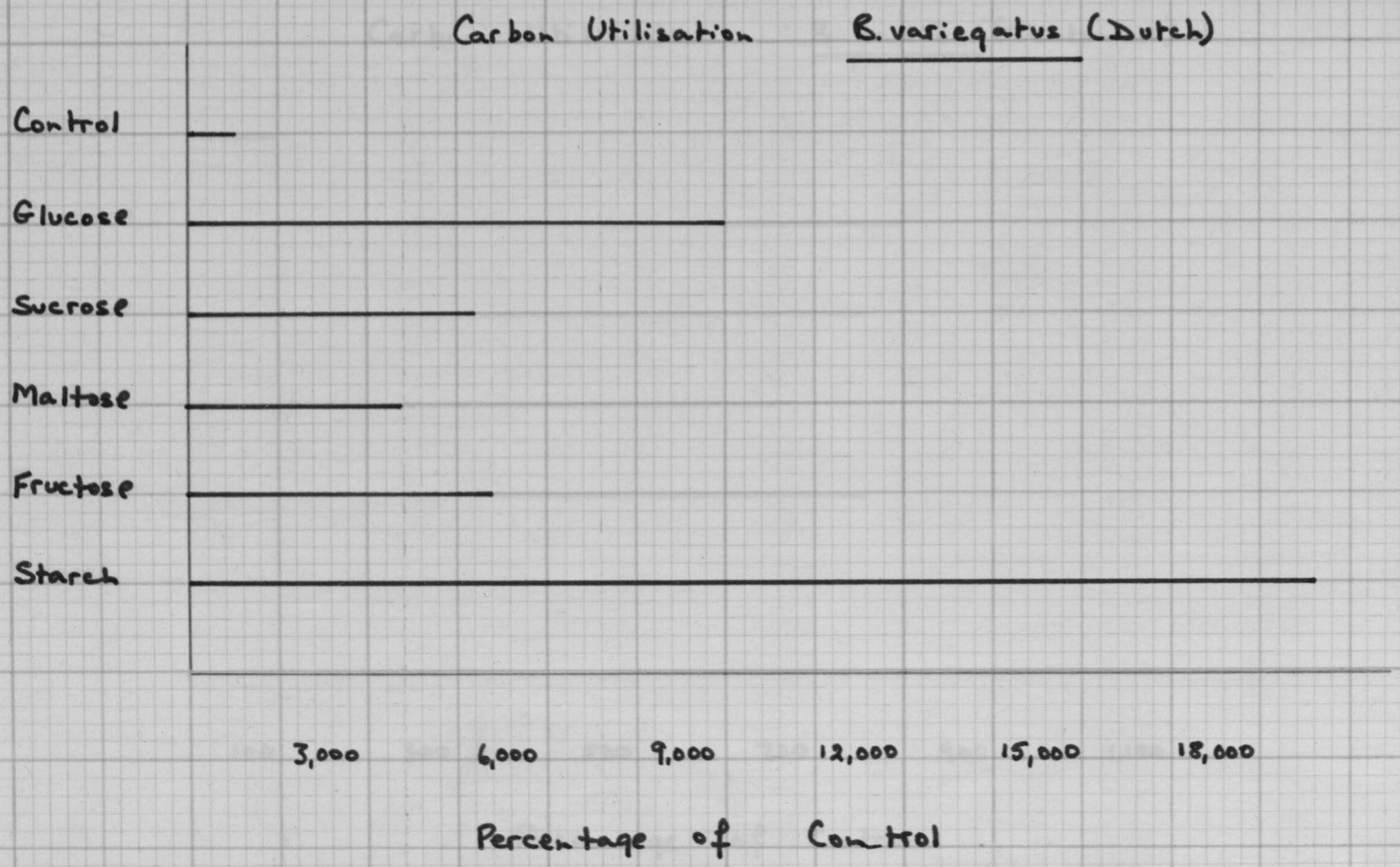

Carbon Utilization
B. badius (Dutch)
Control
Glucose
Sucrose
Maltose
Fructose
Starch
500
1,000
1,500
2,000
2,500
3,000
3,500
Percentage of Control

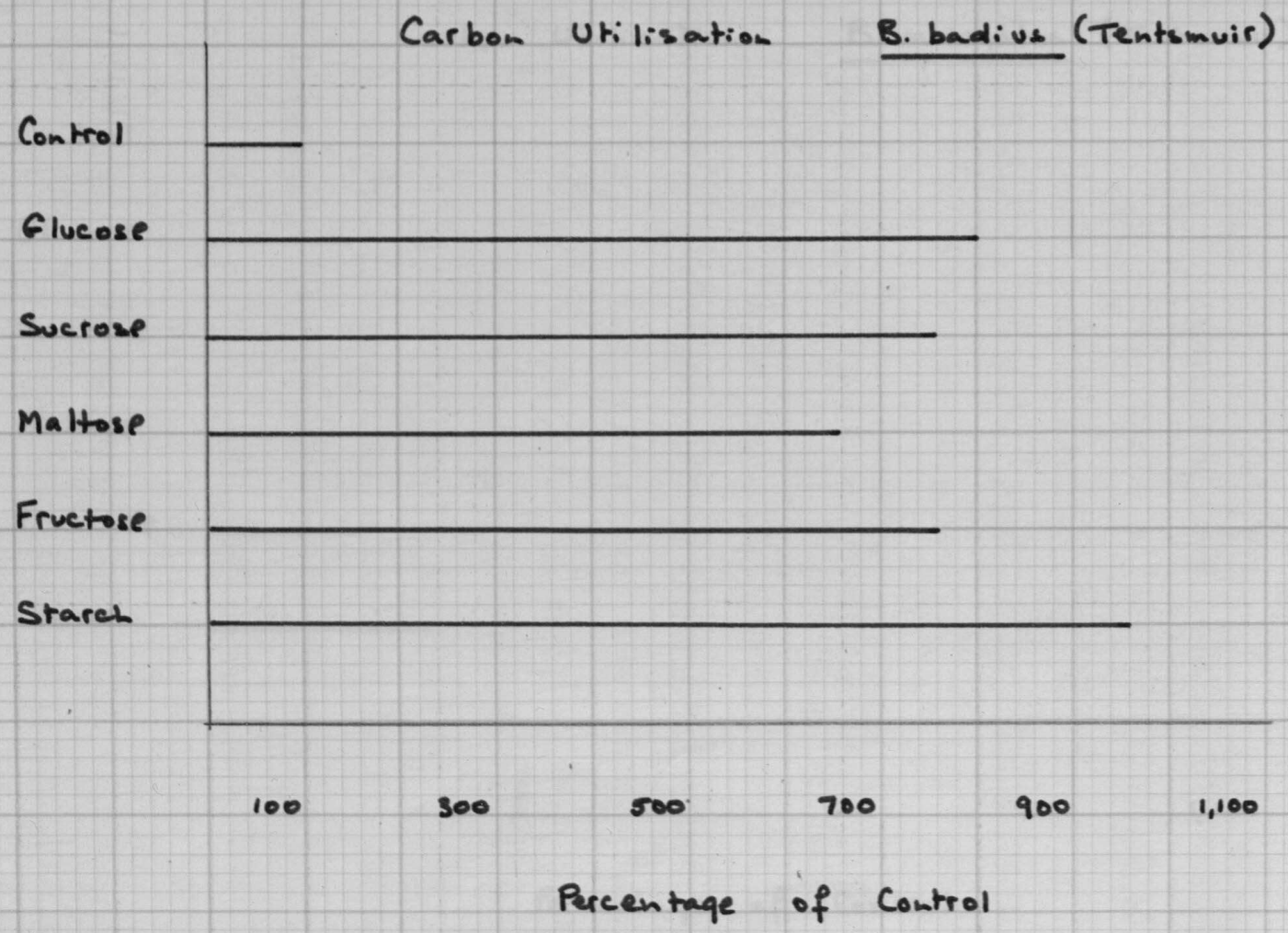

Carbon Utilisation
B. bovinus (Dutch)
Control
Glucose
Sucrose
Maltose
Fructose
Starch
500
1,000
1,500
2,000
2,500
3,000
3,500
Percentage of Control


Carbon Utilisation
B. edulis (Dutch)
Control
Glucose
Sucrose
Maltose
Fructose
Starch
100
300
500
700
900
1,500
Percentage of Control

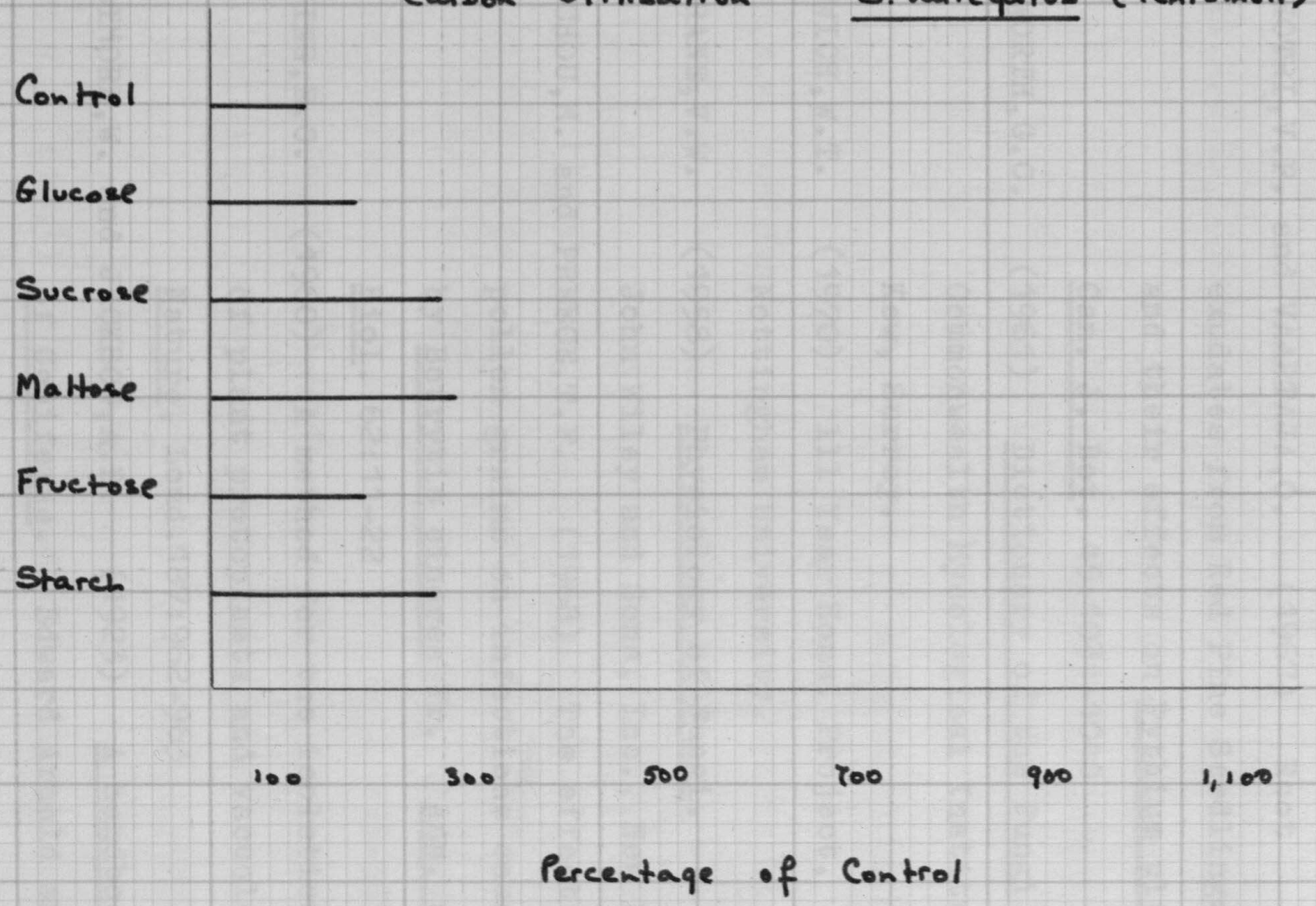
93


Carbon Utilisation B. luteus (Dutch)
Control
Glucose
Sucrose
Maltose
Fructose
Starch
500
1,000
1,500
2,000
2,500
3,000
Percentage of Control


Carbon Utilisation
B. variegatus (Dutch)
Control
Glucose
Sucrose
Maltose
Fructose
Starch
3,000
6,000
9,000
12,000
15,000
18,000
Percentage of Control


Carbon Utilisation B. badius (Tentsmuir)
Control
Glucose
Sucrose
Maltose
Fructose
Starch
100
300
500
700
900
1,100
Percentage of Control

Carbon Utilisation B. variegatus (Tentsmuir)



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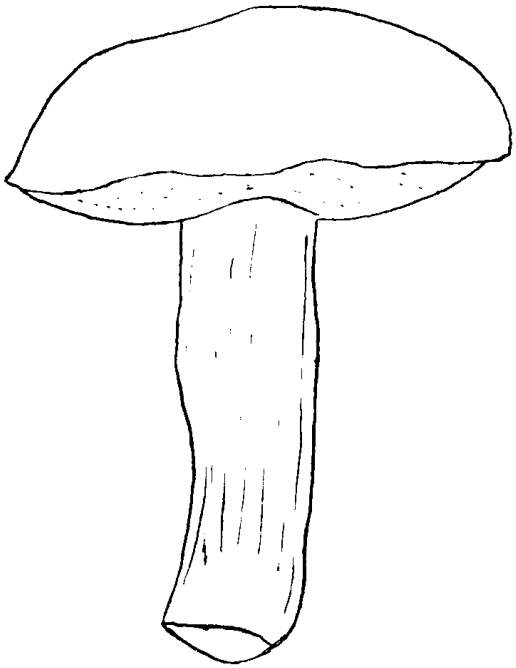
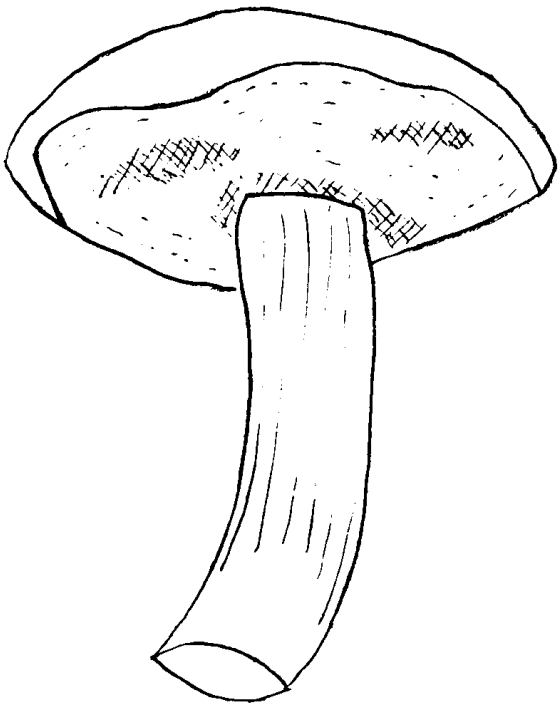
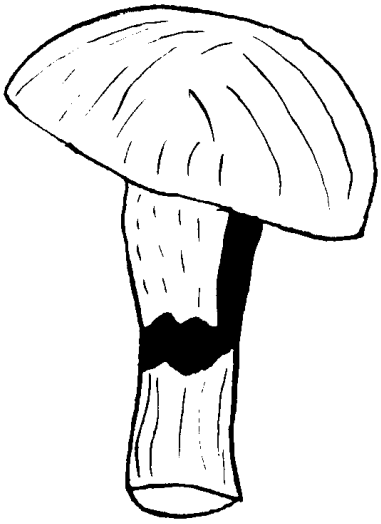


Boletus badius

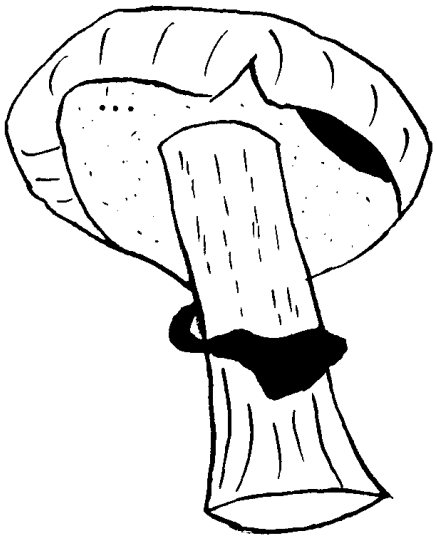


Boletus badius

Figure 1.



Boletus luteus



Boletus luteus

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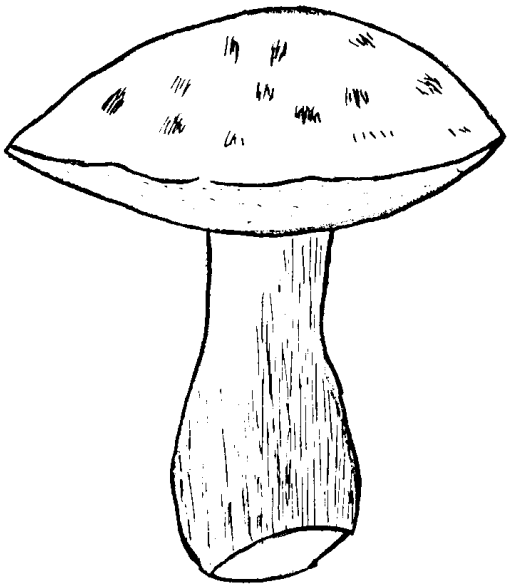
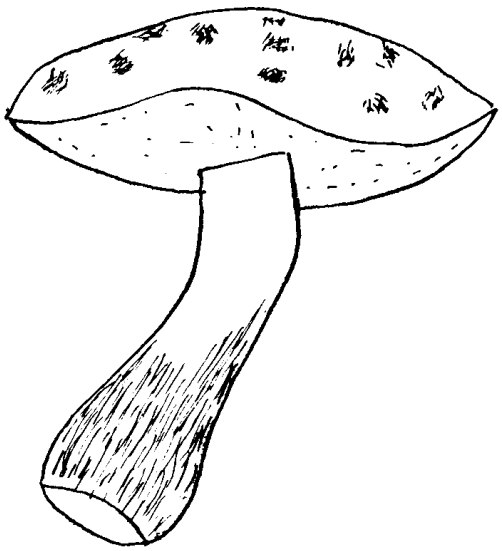
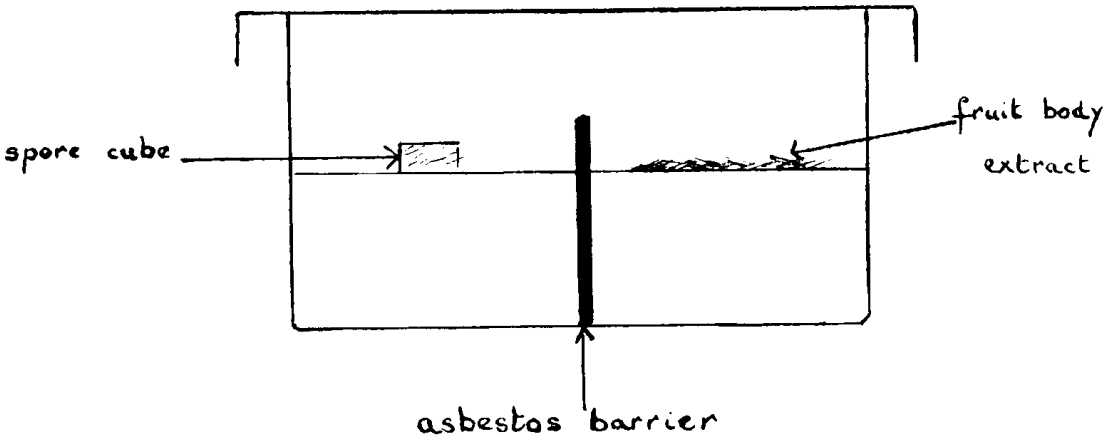


Boletus variegatus



Boletus variegatus

Figure 3.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fruit body
extract
asbestos barrier

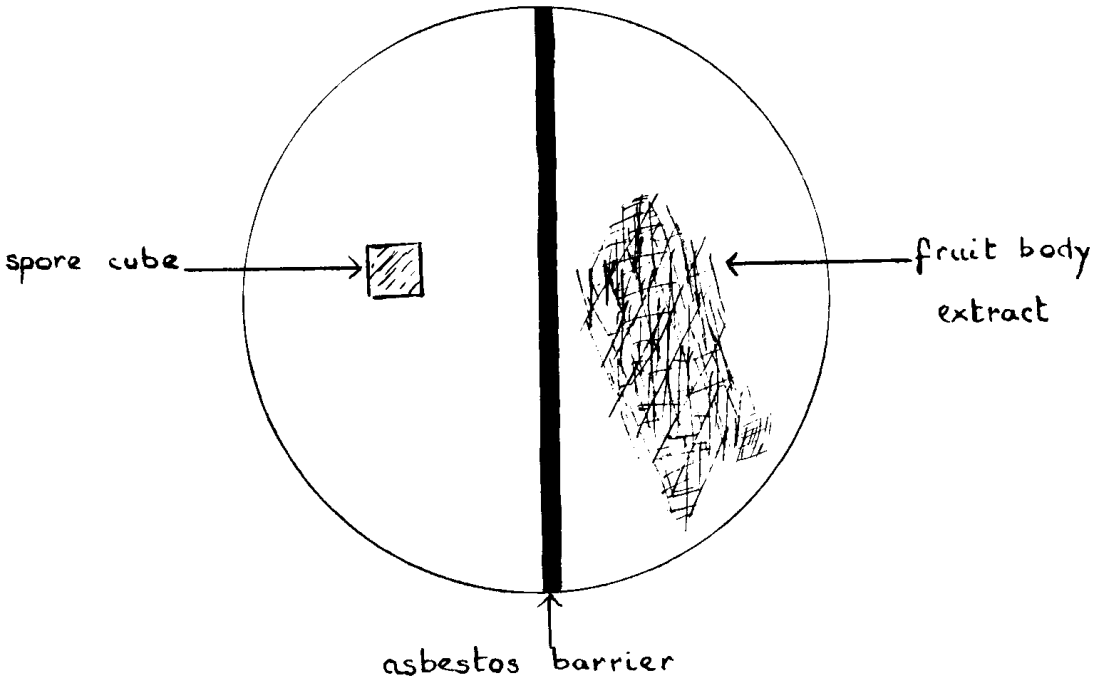
spore cube
fruit body
extract
asbestos barrier

Figure 4.

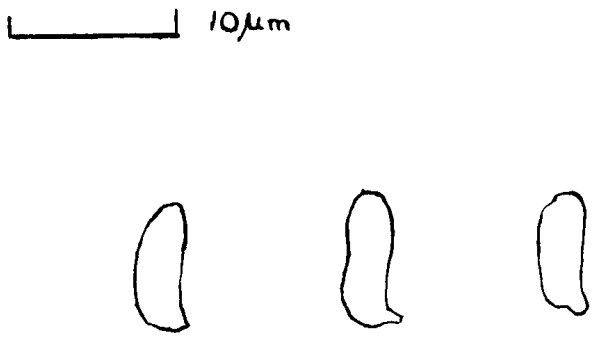

Figure 5 Freshly harvested spores.

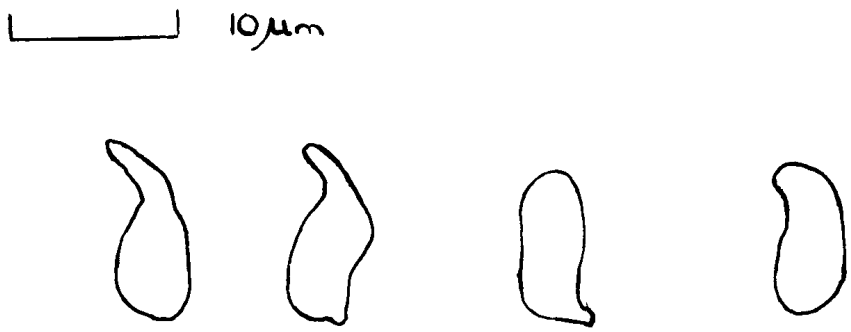

Figure 6 Six weeks old spores, soaked in water for four hours.

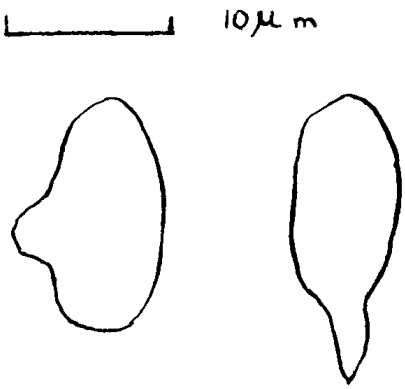

Figure 7 Twenty two weeks old spores, soaked in enzyme solution.

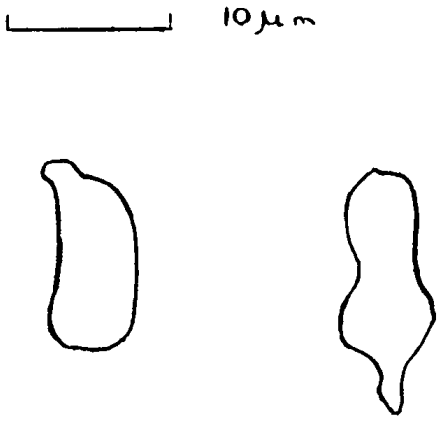

Figure 8 Twenty two weeks old spores, treated with 1% sodium hypochlorite solution.

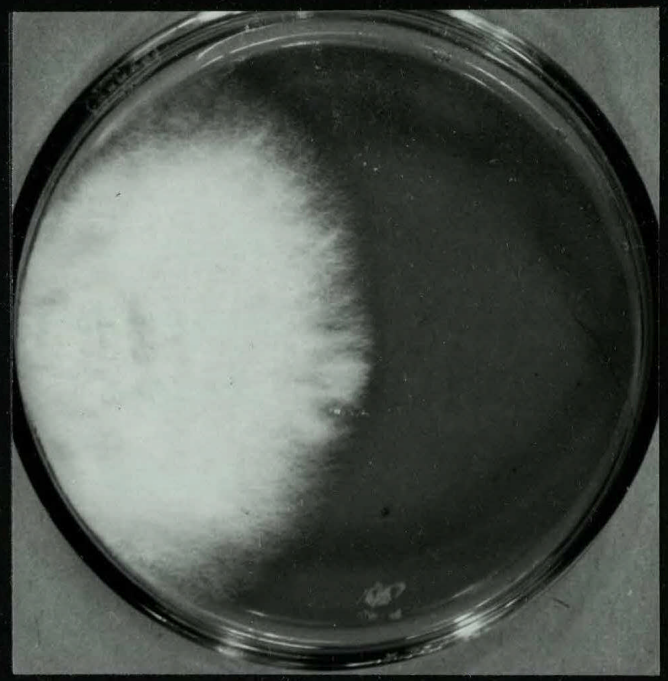

Plate 1. Boletus badius

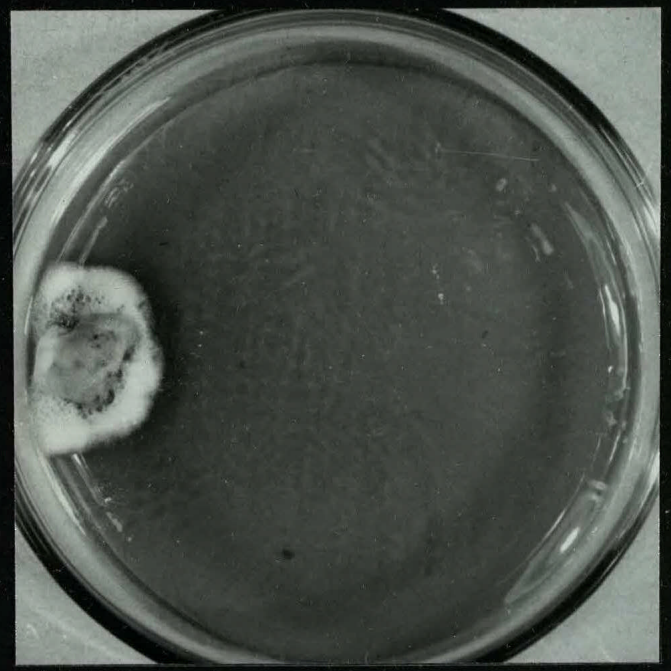

Plate 2. Boletus bovinus

(three weeks after inoculation)

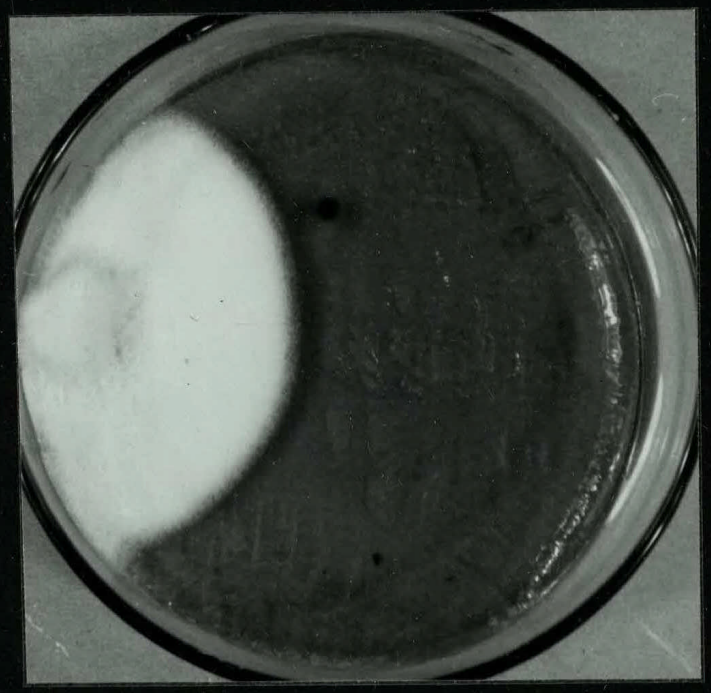

Plate 3. Boletus edulis

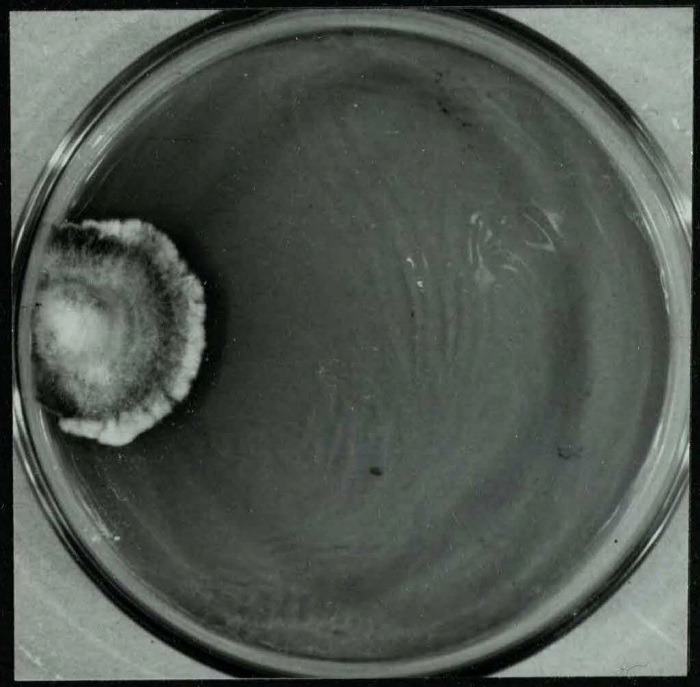

Plate 4. Boletus luteus

(three weeks after inoculation)

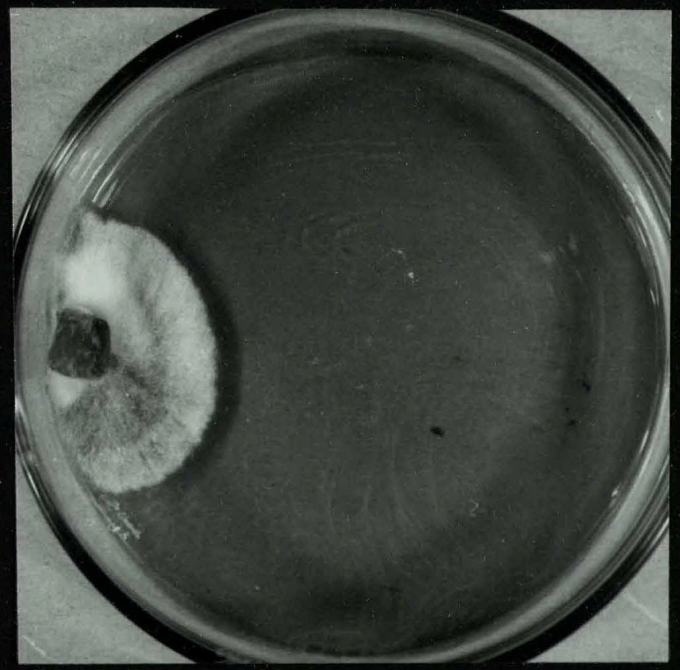

Plate 5. Boletus variegatus

(three weeks after inoculation)