PHYSIOLOGICAL STUDIES OF GROWTH AND DEVELOPMENT IN THE THREE BASIDIOMYCETE SPECIES *LEPISTA NUDA* (BULL. ex FR.) COOKE, *LEPISTA SAEVA* (FR.) ORTON AND *CALOCYBE GAMBOSA* (FR.) SINGER.

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Thesis submitted in fulfilment of the requirements of the degree of Doctor of Philosophy. November 1976

ABSTRACT

Studies were undertaken into the general physiology and fruiting of three wild Basidiomycetes, Lepista nuda (Bull. ex Fr.) Cooke, (wood blewit), Lepista saeva (Fr.) Orton, (pasture blewit) and Calocybe gambosa (Fr.) Singer, (St George's mushroom). The three species were formally grouped together within the genus Tricholoma on overall fruit-body characteristics, but have since been subdivided on the basis of detailed ultrastructure of the fruit-body and spores into the genera Lepista and Calocybe both within the family Tricholomataceae.

C.gambosa is a vernal species, both Lepista species produce fruiting bodies in late autumn. L.nuda occupies a variety of habitats in particular accumulations of partly decomposed leaf litter and rich soils, fruiting in troops under sheltered conditions. L.saeva and C.gambosa generally occupy more open positions in pastures, meadows and fringing woods, often forming 'fairy-rings', and occasionally within woods.

Results from screening a large number of isolates within each species for P-diphenol oxidase and cellulase activity indicate considerable diversity within species and between species. Response to a range of temperature and hydrogen ion concentrations for a smaller number of isolates indicate a more uniform response within the individual species range.

Growth of a restricted number of isolates within each species was determined on a large number of individual carbon and nitrogen sources at a selected carbon:nitrogen ratio. Vitamin, trace metal and fatty acid nutrition was examined for selected strains. Good growth and a wide nutritional diversity was shown by isolates of L.nuda and L.saeva on a wide range of mono-, di-, tri- and polysaccharides, sugar alcohols, hemi-cellulose, cellulose and many nitrogen sources. C.gambosa generally showed restricted growth except where the basal medium was supplemented with natural extracts.

The effect of a range of oxygen and carbon dioxide concentrations was assessed for individual species.

A number of L.nuda and L.saeva isolates were selected for fruiting trials on solid substrates based on their performance in specially constructed tubes and in flasks. Three strains of L.nuda used throughout the survey fruited successfully under artificial conditions, one strain of which almost completed fruit-body maturation on a natural agar medium under simulated enviromental conditions.

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LITERATURE REVIEW

1:0 INTRODUCTION

A review of the literature concerning Lepista nuda , Lepista saeva and Calocybe gambosa reveals that most information available deals with various aspects of the physiology of L. nuda with a relative paucity of information concerning L. saeva and C. gambosa.

The literature from which the information is largely taken deals with comparative studies within the genus Tricholoma (to which these species formally belonged) such as those of Norkrans (1950, 1953) and Rawald (1962 a & b, 1963) and to physiological investigations within fairly heterogeneous groupings of fungi considered to be ectomycorrhizal on certain forest tree species; these include the studies of Lamb (1974) and Lundeburg (1970). L. nuda has also stimulated interest as an edible, rapidly growing and nutritionally unexacting fungus for submerged culture studies and for the production of fruitbodies, it has arroused interest as a fungus which is fairly resistant to contaminants and which produces a wide range of antibiotics (Melik-khachatryan, Abramyan & Gasparyan (1970)).

L. saeva is less frequently encountered and C. gambosa a comparatively difficult fungus to culture,have both received far less attention; this may also be reflected in the fact that neither has been considered to be a mycorrhiza former of any consequence.

The literature as indicated is diverse and neither of these species has singly received detailed attention except L. nuda for a submerged culture study and some pioneering work on fruitbody production by Constantin and Matruchot. The results of individual investigations have been reported factually from data of the individual authors, however, a number are open to serious criticism which is detailed in the following section.

Low yields were obtained by Norkrans (1950, 1953) for L. nuda isolates, dry weights rarely exceeding 15 mg for a 30 day incubation, the best yields with amino-acid or organic acid supplemented

media being about 35 mg following a 35 day growth period, poor yields were also recorded by Modess (1941). Lundeberg (1970) also achieved low yields for his L. nuda isolate of only approx., 60 mg dry weight following 50 days growth on a heterogeneous natural medium in stationary liquid culture.

Very poor growth for C. gambosa isolates is also given by Norkrans (1950, 1953) with yields generally not exceeding 6 mg with 35 days incubation, often falling below 4 mg. The best growth for amino-acid or organic acid supplemented media, for the fastest growing of the three isolates employed in the combined studies, was 47 mg after 60 days incubation.

Rawald (1962 a & b, 1963) relates yields to control values rated at 100 for growth response to a variety of carbon and nitrogen sources and vitamin additions. The controls lacked the substance under study, however, yields generally not exceeding the control value by 200 to 300 per cent, exceptionally 500 per cent for certain of the nitrogen sources, would indicate exceptionally high yields for the controls or overall very low yields for treatments.

The exceptionally low yields shown or indicated by these investigators must cause results for certain of the sections to be treated with reserve as they indicate either the unfortunate choice of extremely slow growing isolates or the use of a medium which is markedly unfavourable to growth. In either event, such phenomena cause a serious detraction from the validity of the results.

Improved yields were given by Lamb (1974) for his isolate of L. nuda on a variety of carbon sources in a synthetic medium containing ammonium chloride as nitrogen source. Many yields were in the 80-125 mg dry weight grouping following 42 days incubation, startling increases in growth of 615, 819 and 1130 per cent were obtained on dextrin, maltose and inulin, as carbon sources when the medium was supplemented with 0·1g/L addition of 'start glucose'.

Votýpka (1972) records much improved growth for L. nuda with rates of 3.5 mm growth per day achieved over malt agar plates.

Very good yields for growth of L. nuda isolates have been reported for submerged culture studies. Reusser, Spencer and Sallans (1958a), Falange, Smith and Rackis (1964) and Falange (1962) record isolates of L. nuda as growing better or equally best among a range of isolates screened for growth on a variety of media. Voltz (1972) in shake flask culture achieved yields between 300 and 418 mg dry weight for the best carbon and nitrogen sources utilised over the 14 day growth period.

Growth on semi-solid media and in stationary liquid culture is probably a better reflection of the adaptive capacity of a fungus in nature, than the growth of strains resulting from the strongly selective pressures encountered under conditions of submerged culture.

The reports concerning fruitbody production by L. nuda were most detailed as performed by Constantin and Matruchot in 1898. Both their attempts, however, and those of the others cited, amount to little more than a transferal of material outside to more sheltered conditions protected from extremes of temperature and desiccation, these studies combined provide almost no information on important facets of culture on solid substrates such as, inoculum potential, growth rate, substrate preference, the factors required for fruitbody formation and the detailed aspects of fruitbody development.

The species names used throughtout the literature survey are those adapted by the individual authors. The nomenclature of these species is dealt with in detail in a later section.

2:0 EFFECT OF PHYSICAL PARAMETERS ON GROWTH.

2:1 Temperature

Norkrans (1950) determined temperature profiles on

glucose-ammonium tartrate minimal medium supplemented with yeast extract over the range 17.5 to 30°C. Temperature optima recorded for T. nudum I and T. gambosum II were 25°C and 20°C at 20, 30 and 30 and 60 days respectively. Growth was prevented at 27.5°C for T. nudum and reduced for T. gambosum. Zadražil (1973) reports temperature optima between 15-20°C for an isolate of L. nuda grown on malt agar.

2:2 Hydrogen Ion Concentration.

Norkrans (1950) reports pH optima for 2 strains each of T. nudum and T. gambosum on a glucose-asparagine nutrient solution, strain II on media where ferric citrate replaced ferric chloride as the iron source. Optima recorded were pH 6.0 at 25 and 20 days for T. nudum strains I and II respectively and pH 5-6.0 and 6.0 for isolates I and II of T. gambosum at 30 days. No shift in pH over the incubation period was recorded, however, on a glucose-ammonium tartrate medium under identical conditions of phosphate buffering where thiamine addition was replaced by 50 ppm of pyrimidine a pH drop of 6.0 to 4.1 and 3.5 was recorded following 30 and 50 days incubation of T. nudum II.
Lundeberg (1970) reports a pH shift during incubation of L. nuda to lower values.

A shift in the hydrogen ion concentration during growth is largely a function of the nitrogen source, an unusually strong pH displacement was recorded by Rawald (1963) where ammonium salts of strong acids were employed for culture of T. georgii and T. nudum isolates.

In submerged culture studies with T. nudum somewhat lower pH optima are recorded, Reusser, Spencer and Sallans (1958b) report optima at pH 3.0 - 5.0 and Voltz (1966) with pH 7.2 and 4.5 at 10 and 20 days incubation respectively.

3:0 UTILISATION OF CARBON SOURCES.

Lamb (1974) conducted a thorough study on the growth of a

range of Basidiomycetes on single carbon sources in a synthetic medium containing ammonium chloride as sole nitrogen source. Best growth was obtained with an isolate of L. nuda on D-glucose, D-mannose, cellobiose and pectin, and moderate development on galactose, mannitol, glycogen and starch. Poor utilisation was achieved with maltose, sucrose, lactose, dextrin and raffinose, however, addition of 0.1 g/L of D-glucose to the medium in the form of 'start glucose' caused fructose, D-galactose, maltose, starch, inulin and glycogen to respond markedly as improved sources of carbon. (Start glucose is a small quantity of added glucose, insufficient to significantly affect yield but sufficient to provide the energy for initial growth and the induction of enzymes for metabolism of sugars that cannot readily be assimilated by the organism). Generally the hexitol, L-rhamnose and the sugar alcohols erthritol, inositol, sorbitol and the corresponding monosaccharids D-erythrose, L-sorbose and D-arabinose permitted little development.

Rawald (1962b) for L. nuda gives best yields for fructose and xylose. Dry-weights obtained for D-galactose, D-glucose, and sucrose were good, growth was poor on D-arabinose and comparable to a carbon-free control on cellulose and pectin. Yields obtained by Rawald for an isolate of L. personata are best for D-xylose, D-galactose, D-glucose, and fructose, moderate growth was obtained with sucrose and starch, poor development on lactose, cellulose and pectin. Empirical comparisons were made for polysaccharides on solidified media using an index of mycelial extension and density, yields for mono-and disaccharides were recorded as dry-weights following growth on a synthetic medium containing ammonium carbonate as sole nitrogen source.

Wilson (1970) recorded growth of T. personatum on cellobiose, glucose and maltose solidified media and demonstrated typical highly branched 'colonial' morphology on cellobiose and similar growth on glucose and maltose; the carbohydrate source was demonstrated to influence activity of cell wall hydrolysing enzymes and hyphal branching.

Norkrans (1950) gives best growth for T nudum on starch,

sucrose, maltose, lactose and fructose respectively. Xylose, raffinose, mannose, and inulin also enabled good growth, poor development only being obtained with galactose. Addition of 'start glucose' (0.5 g/L) to starch and inulin did not significantly increase yields.

Reusser, Spencer and Sallans (1958 a) selected T. nudum from among 10 Ascomycetes and Basidiomycetes grown in submerged culture on synthetic, molasses and waste sulphite media (effluent from the sulphite process in paper pulp manufacture), on the basis of high yields and high protein content; grown in stationary cultures on a synthetic ammonium sulphite medium the T. nudum isolate utilised 25 sources of carbon listed in order of decreasing yield of dry-matter; pyruvate, xylan, sucrose, maltose, D(+) mannose, raffinose, D-arabitol, L(+)-rhamnose, D-galactose, D-fructose, D(+)xylose, dextrin, glucose, D-ribose, cellobiose, sorbitol, L(+)arabinose, starch (soluble), dulcitol, mannitol, inulin, salicin, lactose, L(-)sorbose and cellulose.

Reusser, Gorin and Spencer (1960) showed that T. nudum grown on sucrose (4%), molasses and raffinose media, preferentially utilised the glucose leaving fructose in the fermentation broth. The fructose could be purified and recovered. They suggested that glucose may inhibit fructose assimilation. Good growth was also recorded on galactose and melibiose.

Worgan (1968) reports obtaining good growth of T. nudum on a medium containing xylose as sole source of carbon.

In shake-flask culture on the medium of Humfeld and Sugihara (1952), Voltz (1972) reports growth of an isolate of L. nuda on a large number of mono-, di-, and polysaccharides also including a range of organic acids and miscellaneous carbon sources. Outstanding dry-weights were obtained with D-mannose and cellobiose, good yields with D-glucose, D-fructose 1,6 diphosphate and pectinol-A. D-fructose, xylan, pectin and galactouronic acid gave moderate growth, poorer development being obtained with DL-arabinose, maltose and raffinose. L-sorbose, and α & β lactose,

sucrose, glycogen, starch, tannic acid, i-inositol, D-mannitol, D-sorbitol and casein gave poor yields. Dry-weights similar to the carbon free control value were obtained with D-arabinose, D-ribose, D-xylose, citric acid, DL-malic acid, oxalic acid, pyrogallic acid, resorcyclic acid, succinic acid, D-tartaric acid and D-glucosamine hydrochloride.

3:1 Growth on Cellulose and Lignin.

Norkrans (1950) records growth on and clearing of opaque cellulose agar in Petri-plates by isolates of T. nudum and T.gambosum.

Lindeberg (1946) and Norkrans (1950) cultured isolates of T.gambosum and T.nudum plus T.personatum respectively on sterilised Glyceria straw in flasks for periods of 165 days and 8 months. T.gambosum decomposed 16.1% of the dry-matter and 18.7 and 24.2% of the lignin and cellulose content. T. nudum removed approximately 37% of the dry-matter and decomposed about 23 and 65% of the lignin and cellulose. T.personatum decomposed approximately 30% of the dry-matter and about 3 and 66% of the lignin and cellulose. T. personatum only demonstrated a capacity to attack lignin where the flask contents were supplemented with either 25 or 125 mg glucose. However, Norkrans reports a positive Bavendamm test for this species.

In in vitro studies on cellulolytic enzyme preparations from T. nudum, Norkrans (1950) demonstrated an optimum temperature between 30 and 37°C and considerable heat stability - total inactivation occurring only at 100°C. The enzyme preparation exhibited a pH optimum in the range of 4.5 to 5.0 and maximum stability over the range 4.0 to 6.0, however, it was not readily inactivated by pH alteration over the range 1.2 to 10.0 prior to readjustment to pH 5.0 Cellobiose at 0.03% caused in excess of 50% inhibition of cellulolytic ability, total replacement of cellulose with cellobiose permitted cellulolytic activity amounting to 20-30% of that of the pure cellulose control value. This repression was also shown by glucose and a number of sugars.

Growth on lichenin (lichenin prepared from Icelandic moss,

Cetraria islandica is 73% glucose 1,4 linked and 27% in a 1,3 position; this produces an irregular chain giving lichenin an open easily attacked structure) and cellulose, the latter with and without 'start glucose' addition, was investigated for T. nudum and T. gambosum by Norkrans (1950). In both cases lichenin was more readily attacked and served as a better carbohydrate source. 'Start glucose' did not assist utilisation of the cellulose for either species.

Norkrans (1950) records being unable to show cellobiose, mannan, starch or maltose splitting enzymes in crude enzyme preparations from T.nudum following incubation periods of varying lengths. However, enzymes attacking native and regenerated cellulose, salicin, sucrose and raffinose were indicated by the presence of reducing breakdown products.

Lundeberg (1970) reports total inability of his isolate of T. nudum to produce cellulase, together with trace pectinase following 50 days incubation, but strong proteinase and laccase activity on a medium containing tannin, cellulose, pectin, malt extract and bone meal.

T. nudum was shown to grow well in submerged culture on waste sulphite liquor, a waste product resulting from the removal of lignin in paper-pulp manufacture (Reusser, Spencer and Sallans 1958 a & b), however, they report that it was unable to utilise the lignin hydrolysates vanillin, syringealdehyde and p-hydroxybenzaldehyde when grown in stationary culture.

4:0 UTILISATION OF NITROGEN SOURCES.

Norkrans (1950) reports yields of T. nudum I on a glucose-salts basal medium as best with asparagine, urea and ammonium tartrate. Good growth was recorded on glycine, alanine and ammonium hydrogen phosphate. Moderate yields were obtained on potassium nitrate, ammonium sulphate and ammonium chloride. Leucine gave poor development.

Very poor growth was obtained with T. gambosum I with extended

incubation under the same conditions. Yields did not exceed 3.3 mg. Potassium nitrate did not appear to be utilised, otherwise the figures were not significantly different over the range of nitrogen sources listed for T. nudum I.

Lundeberg (1970) reports best yields of T. nudum obtained on asparagine, with moderate yields on ammonium tartrate and glycine. Yields were not obtained in excess of the nitrogen-free control value with potassium nitrate, acetamide, diethylamine, D-glucosamine, L-proline or pyridine. Potassium nitrite totally inhibited growth.

Rawald (1963) records the best yield for T. nudum on propionamide; good growth was obtained relative to the control with asparagine, glycine, ammonium tartrate, urea and peptone, these values were just superior to those obtained on casein hydrolysate and leucine. Average yields were given on triethylamine, sodium nitrate, potassium nitrate, and valine. Poor yields were produced with valeric acid amide, sodium cyanide and sodium nitrite. Ethyl cyanide, ammonium carbonate and acetonitril gave yields only slightly better than the nitrogen free control. Ammonium chloride gave yields equivalent to the control value, sodium rhodanid proved inhibitory to growth.

Lack of growth obtained by Lundeberg (1970) on acetamide contrasts with the excellent yields obtained with propionamide indicated by Rawald for T nudum. Growth of T. nudum, though small, on sodium nitrite was unusual.

Dry-weights obtained with C.georgii by Rawald were superior with peptone, good yields were given on asparagine, propionamide and ammonium tartrate in relation to the control value. Casein hydrolysate was an improvement on acetonitril, leucine and betaine hydrochloride, which are indicated as better than average sources of nitrogen for this species. Sodium cyanide, triethylamine and collectively urea, ethylcyanide and potassium nitrate were respectively moderate yielding nitrogen sources. Valeric acid amide, ammonium chloride and ammonium carbonate produced values

falling to and similar with that of the nitrogen free control. Sodium rhodanid and sodium nitrite caused inhibition of growth.

Voltz (1972) records yields obtained from growth on amino-acids and related nitrogen sources substituted into Humfield's and Sugihara's medium (1952) for L. nuda grown in shake-flask culture.

Glycine produced yields superior to all nitrogen sources tested. Peptone and DL-alanine at approximately half this value yielded well, L-glutamic acid, DL-methionine and aspartic acid produced dry-weights, at about a quarter of that obtained with glycine, were fair nitrogen sources. L-Asparagine and DL-valine gave low yields. L-proline, creatine, betaine hydrochloride only slightly bettered the nitrogen-free control value which equalled yields on DL-isoleucine, DL-threonine & DL-tryptophane . L-cystine, D-histidine and cysteine hydrochloride caused growth inhibition.

In comparison with the ammonium and nitrate nitrogen sources tested, ammonium bicarbonate and urea yielded somewhat higher than peptone, ammonium tartrate supported poor growth similar to L-asparagine. Magnesium nitrate and ammonium nitrate gave slightly improved yields to that of the nitrogen-free control. Values almost identical to that of the control were given on sodium nitrate, potassium nitrate, ammonium sulphate and ammonium chloride. Inhibition of growth was encountered with cobaltous ammonium sulphate, ammonium citrate, cupric nitrate, cobaltous nitrate and uranium nitrate as nitrogen sources.

Reusser, Spencer and Sallans (1958 b) list nitrogen sources utilised for a T. nudum isolate on a synthetic glucose-salts medium in stationary cultures. Double the quantity of nitrogen was added for racemic mixtures where it was assumed only the L-isomers were utilised. Organic nitrogen sources utilised were : L-asparagine, DL-alanine, glycine, DL-serine, L-arginine, L-proline, C-threonine, L-glutamine, DL-valine, L-tyrosine, L-leucine, DL-leucine, DL-isoleucine, DL-alanine, L-histidine, DL-lysine L-tryptophane, DL-∝-aminobutyric acid, DL-glutamic acid, DL-methionine, DL-aspartic acid and DL-phenylalanine, L-taurine was not utilised.

Inorganic nitrogen sources utilised were potassium nitrate, ammonium nitrate, ammonium tartrate, urea, ammonium phosphate (dibasic) ammonium phosphate (monobasic), ammonium chloride and ammonium sulphate.

Varying the ammonium tartrate concentration of the standard glucose-ammonium tartrate basal medium, between 0.025 and 1 per cent was found to increase crude protein levels from 18 to approximately 50 per cent and decrease fat from 24 to 4 per cent.

Reusser, Spencer and Sallans (1958 b) and Rawald (1963) both note poor growth where the ammonium salt of a strong acid was used, which resulted in a rapid fall in pH to inhibitory levels. Reusser et al., (1958 b) found that adopting a mediumof high buffering capacity enabled satisfactory yields to be obtained on compounds such as ammonium sulphate.

5:0 VITAMIN REQUIREMENT FOR GROWTH.

Norkrans (1950) for T. nudum II on a glucose-ammonium tartrate basal medium records strongest development on yeast extract, only approximately two-thirds of this extent of growth was attained on a synthetic yeast-extract vitamin 'M'. (Vitamin mixture 'M' was composed of biotin, thiamine, calcium pantothenate, "folic acid", nicotinamide, p-aminobenzoic acid, pyridoxine, riboflavin, choline and inositol). Growth with aneurin, aneurin-biotin, aneurin-inositol and aneurin-biotin-inositol produced similar yields at less than half that attained on yeast extract and a third below that produced with the synthetic vitamin mix.

T. nudum I did not show significant stimulation of growth on vitamin-mixture 'M' compared to growth on aneurin or a combination of aneurin, biotin and inositol. Removal of calcium pantothenate from the synthetic vitamin mix caused a reduction in yield to slightly more than half that obtained with 'M'. Removal of choline, "folic acid" nicotinamide or riboflavin singly from the mixture 'M' did not cause any discernable alteration in yield, subtraction of p-aminobenzoic acid and pyridoxine caused a slight elevation in yield relative to 'M'.

Vitamin mixture 'M' almost doubled the dry-weights obtained with T. gambosum with aneurin or collectively aneurin, biotin and inositol. Calcium pantothenate caused a drop in yield to a value in slight excess of that obtained with aneurin. Exclusion of pyridoxine, p-aminobenzoic acid and nicotinamide from the vitamin mix slightly increased yield/ compared to the complete mix. Exclusion of choline, "folic acid" and riboflavin did not significantly alter yield.

Growth of T. nudum I and T. gambosum I isolates on the basal medium only took place where intact aneurin, or the pyrimidine moity of the two thiazole and pyrimidine moities comprising aneurin, was present.

In an investigation of the effects of suboptimal amounts of pyrimidine on growth of T. nudum I and II and T. gambosum II isolates, a direct proportionality was shown in the amount of growth obtained and the corresponding quantity of the pyrimidine moity supplied over the range 0, 25, 50, 100 µµmol pyrimidine. The addition of 4 m µmol of pyrimidine following cessation of growth in the 50 µµmol series caused an immediate resumption of strong growth.

From maximum yields of T. nudum isolates I & II in the 50 µµmol series the mycelium: pyrimidine quotient (M/P) was calculated as 1.0 and 1.8×10^6 respectively or on a molecular basis 2.2 and 3.7×10^8 respectively.

Kögl and Fries (1937) for an isolate of T. nudum obtained almost absence of growth in the vitamin free control and with the addition of biotin and inositol. Aneurin and biotin, inositol and aneurin produced growth to a comparible extent.

Rawald (1962) studied the vitamin requirements of C. georgii, L. personata and several strains of L. nuda utilising a glucose-ammonium carbonate basal medium.

L. nuda produced best yields supplemented with pressed

brewers and bakers yeasts respectively, which stimulated growth considerably more than biotin, which was individually superior to thiamine, calcium pantothenate and p-aminobenzoic acid. Pyridoxine hydrochloride and niacinamide produced just significantly more growth than the vitamin-free control.

For six strains of L. nuda addition of vitamins individually produced just significantly greater growth than that obtained for the control with thiamine (in 2 strains) and biotin, pyridoxine hydrochloride and inositol (in 1 strain each).

Growth of L. personata was best with thiamine, yields declined to the control value respectively with pressed brewers yeast, pressed bakers yeast and calcium pantothenate followed by a mixture of vitamins in the B complex then inositol and finally biotin which was significant with respect to the vitamin -free control value.

C. georgii showed greatest growth on thiamine addition, growth in excess of, but declining to that of the control value was obtained with pressed bakers yeast followed collectively by pressed brewers yeast and calcium pantothenate.

Reusser, Spencer and Sallans (1958 b) for their work with T. nudum on synthetic glucose-ammonium sulphate medium employed thiamine, pyridoxine and p-aminobenzoic acid as vitamin additions.

6:0 EFFECT OF AMINO-ACIDS, AMIDES AND ORGANIC ACIDS AS GROWTH FACTORS.

Norkrans (1950) investigated the effect of casein hydrolysate supplement (25 mg/L) to the glucose-ammonium tartrate basal medium also the effect of 21 different amino-acids added singly inthe proportion that they occured in the casein hydrolysate preparation calculated on the basis of 25 mg. With T. gambosum growth was most strongly promoted with glutamic acid, hydroxyproline and histidine. Tyrosine and threonine markedly inhibited growth. Casein hydrolysate did not accelerate the growth rate of T. nudum, tryptophane, valine and arginine caused an increase in dry-weight above

that of the control. Tyrosine, histidine and isoleucine caused inhibition of growth, the remaining amino-acids had hardly any effect. In neither case did the level of growth promotion indicate a requirement for essential amino-acids by these isolates.

Norkrans (1953) examined the effect of aspartic and glutamic acids and their corresponding keto-acids and half amides as growth stimulating substances for isolates of T. nudum and T. gambosum III especially during the initial stages of growth. Substances were added at a concentration of 10 μ mols per flask (0.4 m mols/L) to either a glucose-ammonium tartrate or glucose--ammonium hydrogen phosphate synthetic medium. Asparagine and glutamine produced the greatest response with T. gambosum giving relative growth values of 525 and 375 compared to a control of 100 after 30 days. Oxaloglutarate proved to double dry-weight, whereas its amino-acid glutamic acid had no effect.

Casein hydrolysate at 0.184g/L was more beneficial than asparagine in its effect over the 60 day incubation period. The dipeptide glycyl-glycine promoted tripled mycelial production of T. gambosum whereas singly glycine exerted only slight effect. This finding, together with the previously observed stimulative effect of asparagine and glutamine, is postulated by Norkrans to reflect certain difficulties in peptide synthesis by this species.

In a separate experiment with T. gambosum where additions of 250, 50, 10 and 2 μmols of the respective substances were used, maximum growth effect was obtained with 10 μmol additions in the aspartic acid series but 50 or 250 μmol in the glutamic acid series.

These substances produced only a slight growth promotive effect on the T. nudum isolate, values not in excess of 18 mg were achieved, the keto-acids appearing to be more stimulatory than either of their corresponding amino-acids or amides.

6:1 Leaf litter extracts as sources of Growth Factors.

Norkrans (1944) recorded that aspen-litter extract at low concentrations exercised a growth-promoting influence on several Tricholoma species, however, this effect was reversed at higher concentrations with growth inhibition apparent.

Melin (1944) reported leaf-litter extracts of maple, beech, oak, aspen and pine stimulated litter-decomposing hymenomycetes to a particularly high degree. The litter decomposers were stated to be insensitive to water soluble inhibitory substances contained in the litter which inhibited or prevented growth of many soil hymenomycetes, particularly mycorrhizal fungi.

7:0 INFLUENCE OF LEAF-LITTER ASH AND METAL IONS.

Norkrans (1950) did not report growth stimulation of either T. nudum or T. gambosum isolates with zinc or manganese addition to a basal medium containing potassium dihydrogen orthophosphate, magnesium sulphate, sodium and ferric chloride as inorganic constituents.

Addition of calcium at 9 mg/L caused an increase in yield, added at four times this amount growth equalled that at this higher level of calcium with zinc and manganese additions and also with aspen foliage litter plus this metal ion combination.

The effect of ash alone, also the higher level of calcium, were particularly stimulatory to the growth of T. nudum. The growth promotory effect of the ash addition was considered to be largely due to its calcium component, an element which is well documented for its ability to nullify the inhibitory action of excessive levels of other metal ions in the culture medium.

8:0 FATTY ACID COMPOSITION AND ASPECTS OF BIOSYNTHESIS.

Following observations on variation in fat content of T.nudum

mycelium by Reusser, Spencer and Sallans (1958 b) resulting from alteration to the nitrogen level of the glucose-ammonium tartrate medium employed for submerged culture studies (levels of 4.2 to 24.4 per cent fat at C:N ratios of 16 and 384:1 respectively), several detailed studies were instigated into the composition and mode of synthesis of fats by T. nudum in a low nitrogen medium.

Leegwater, Youngs, Spencer and Craig (1962) investigated the fatty acid composition of the mycelium by thin layer, column and gas-liquid chromatography in conjunction with a number of additional novel techniques.

At two days incubation, while still in the lag phase of growth, this revealed the neutral lipid fraction (the larger component) to contain as the major fatty acids palmitic, oleic and linoleic acid; stearic acid constituted a lower proportion. Myristic, palmitoleic and linolenic acids were represented in small amounts.

Linoleic acid was the major fatty acid of the phospholipids with palmitic, stearic and oleic as minor components, myristic, palmitoleic and linolenic were present in a small quantity.

By contrast in 1-day-old cultures the composition of both fractions was distinctly different, with linoleic acid the main fatty acid, linolenic acid being also present as a major component of the triglycerides during this period, indicating that linoleic acid, and its closely related fatty acid, linolenic acid play a special role in the young developing mycelium.

Analysis of the major components at 4 days incubation revealed the neutral lipids to consist largely of triglycerides with trace quantities of ergosterolesters, free fatty acids, a higher level of ergosterol plus a number of unidentified non-saponifiable compounds.

The phosphlipids were above half phosphadyl-choline in

composition and approximately a quarter phosphadyl ethanolamine. Phosphatidyl serine and phosphatidic acid plus an inositol containing phospholipid were present in minor amounts.

In radioactive tracer studies utilising glucose-U-C^{14} Leegwater and Craig (1962) showed T. nudum mycelium incorporated label into the linoleic fraction of the triglycerides after a considerable lag compared to that of palmitic, stearic and oleic fractions. Incorporation of linoleic acid into the phospholipids was in the same proportion to that of the other major fatty acids, this led to them postulating possibly separate fatty acid pools for neutral and phospholipid synthesis. Although a pathway of oleic acid conversion to linoleic acid was established, they concluded that this anomalous labelling pattern of linoleic acid indicated the involvement of, as yet, unidentified pathways, also, that the rate and order of fatty acid synthesis is largely dependant on experimental conditions.

8:1 Growth Response to Aliphatic Alcohols and Fatty Acids.

The importance of fatty acids and alcohols in the nutrition of isolates of L.nuda, L.saeva and C.gambosa, has not received consideration though has proved of considerable significance in the physiology of certain fungi. Information concerning those basidiomycete species which have elicited a marked growth response is summarised in this section.

Wardle and Schisler (1969) for single-spore isolates of the cultivated mushroom, Agaricus bisporus reported growth stimulation of between 4 to 6 times that obtained on the xylose-asparagine basal medium following the addition of a range of animal and vegetable oil and fat supplements, best growth was obtained with cottonseed and corn oils.

Ethyl and methyl esters of oleic and linoleic acid added at levels similar to that found in cottonseed and corn oils produced a growth response to a similar extent. The mycelial growth response did not show a linear relationship, suggesting that more than one factor was responsible for the growth stimulation.

The fatty acids myristic, arachidic and hexadecenoic were absent in some of the oils tested which elicited a dry-weight increase similar to those oils containing these fatty acids, thus these were not considered responsible for the increased growth.

Weinhold (1963), Weinhold and Garraway (1966) have demonstrated the importance of ethanol and related low molecular weight alcohols in stimulating growth and rhizomorph formation in Armillaria (Armillariella) mellea on a synthetic glucose-asparagine medium.

Ethanol, 1 - propanol and 1-butanol stimulated utilisation and rhizomorph development on a range of simple sugars and amino-acids, which otherwise produced little or no mycelial development. Alcohol also served as good sole carbon source giving growth equal to glucose plus ethanol supplement. Acetate supported some mycelial growth but no rhizomorphs.

In a study with ethanol-C^{14} Garraway and Weinhold (1968) showed that more of the radioactive carbon appeared in the lipid fraction than elswhere.

Moody and Weinhold (1972) demonstrated that a range of monocarboxylic acids, free fatty acids and plant lipids stimulated rhizomorph formation of A.mellea. The order of stimulation shown by the vegetable oils suggests that the unsaturated fatty acid fraction contributed to their activity. The effectiveness of the monocarboxylic acids and unsaturated fatty acids was shown to be proportional to chain length, toxicity of the latter increased in direct relation to the number of double bonds.

Where neutral lipids or fatty acids were sole sources of carbon, no rhizomorph development took place, indicating that they served as growth promoting substances and not sources of carbon. The organic acids and fatty acids were active at much lower concentrations than the plant lipids or triglycerides tested.

Sortkjaer and Allermann (1972) established that both

ethanol and acetate were effective in inducing rhizomorph development in A.mellea. They established a continuous requirement for ethanol. The use of disulfiraminhibited the action of ethanol but not that of acetate, suggesting that A.mellea possesses alcohol dehydrogenase activity. It was concluded/ that the pathway ethanol→acetaldehyde→acetate→fatty acids is in operation (the/ alcohols being converted to their respective acids) and which exerts a regulating effect on the metabolism of glucose.

Allermann and Sortkjaer (1973) reported vigorous rhizomorph formation and strongest growth stimulation of 2 isolates of A.mellea and an isolate of the closely related fungus Clitocybe geotropa with ethanol, 1-propanol and 1-butanol. Secondary branched alcohols, 2-propanol and 2-butanol showed only slight stimulatory capacity, primary branched alcohols, iso-butylalcohol and iso-amylalcohol exert various degrees of stimulation, tert-butyl alcohol and glycerol induced vigorous rhizomorph formation in all three isolates.

Fries (1961) showed that nonanal (pelargonaldehyde) an oxidation product of oleic acid, stimulated the growth of a number of fungi in particular wood decomposers. The closely related homologues to nonanal, the aliphatic aldehydes with 6 to 8 carbon atoms, as well as their corresponding alcohols were also active but to a lesser degree than nonanal.

Yusef (1953) reported that the growth of Polyporus schweinitzii was improved by the addition of oleic acid.

9:0 EFFECT OF OXYGEN AND CARBON DIOXIDE TENSION ON GROWTH.

Carbon dioxide markedly affects the mycelial growth of Agaricus bisporus. Data obtained on the influence of a range of carbon dioxide concentrations (Tschierpe, 1960 & 1972, Nair 1972) shows that mycelial growth is strongly inhibited by concentrations above 15% and prevented at 32%. Maintaining nitrogen constant at 79%, Nair (1972) showed that growth inhibition became significant (1% level) at 6.6% carbon dioxide. Data was obtained using a diffusion column.

San Antonio and Thomas (1972) have shown a growth optimum for hyphal fragments of A.bisporus on a synthetic agar medium at concentrations of carbon dioxide in the order 0.3-0.6%.

Le Roux (1962), Rast and Bachofen (1967) and Bachofen and Rast (1968) utilising radioactive carbon dioxide have demonstrated metabolic pathways whereby A.bisporus is capable of carbon dioxide fixation and utilisation. Le Roux (1972) has shown A.bisporus strands are better able to utilise carbon dioxide than dispersed hyphae.

Griffin and Nair (1968) using the diffusion column technique (Griffin, Nair, Baxter and Smiles, 1967) have clearly shown the effect of carbon dioxide and oxygen tension over the range of 0-15% for growth and sclerotium formation of Sclerotium rolfsii. Maximal growth was attained until oxygen concentrations lower than 4% were reached. Altering the levels of carbon dioxide alone produced almost identical results to those given by an inverse gradient of carbon dioxide and oxygen tension (nitrogen level maintained oonstant), showing that growth was roughly inhibited in direct proportion to the level of carbon dioxide in the atmosphere of the culture tubes.

Louvet and Bulit (1964) record a halved growth-rate for Sclerotinia minor at 4% carbon dioxide.

Burges and Fenton (1953) measured the growth of three soil fungi over a range of carbon dioxide concentrations from 0-20% and were able to relate the vertical distribution of the fungi by the level of growth inhibition caused by carbon dioxide. The Penicillium sp., from the upper 5 cm of soil was strongly inhibited, the Zygorrhynchus sp., abundant at 10 cm showed good growth at 20% carbon dioxide, a Gliomastix sp., present through out the soil profile was tolerant of low carbon dioxide tensions.

In general fungi colonising the aerial parts of plants or light soils are less tolerant of high carbon dioxide levels than those growing at depth of soils (Durbin 1955, 1959, Papavizas and Davey 1962).

As pointed out by Griffin (1972), however, the scope of such studies should be limited to the range 0-5% carbon dioxide, because of the unlikely occurrence of levels of this gas above this figure in the normal surface soil atmosphere, although the carbon dioxide levels in water films particularly those adjacent to respiring organs and in water saturated soils may be considerally higher (Griffin 1963). In a consideration of the role of carbon dioxide as a regulator of fungal growth in natural soils, its relative abundance in proportion to carbonate and bicarbonate levels (as determined by the dissociation constant in soils of different hydrogen ion concentration) and the affects of these ions requires to be taken into account.

Tabak and Bridge-Cooke (1968) in a thorough review on the effects of gaseous environments on fungal growth, record fungi as generally capable of vegetative growth at low oxygen tensions.

Wood-decomposing fungi including species of Schizophyllum (Schwalb and Miles 1967), Fomes (Gunderson 1961) and Pleurotus (Zadrazil 1974) may show an adaptive ability to grow at very low oxygen tensions or high levels of carbon dioxide.

10 : 0 ECOLOGICAL ASPECTS OF GROWTH AND FRUITBODY DEVELOPMENT.

Wilkins and Patrick (1940) record the fruitbody numbers of T.gambosum as the total count for six stations on clay and seven stations on chalk. Fruitbodies were collected over the period 27th April to 22nd June. Highest numbers were recorded in late May on clay sites, smaller numbers were given for the chalk sites with an earlier peak in late April and early May. Earlier fruitbody development on the chalk was correlated with a higher water content. Soil temperatures were not considered to differ significantly throughout the year between both stations.

Sporophore numbers of T.personatum are given as a total for the six stations on clay, fruiting was recorded from 22nd October to 2nd January. Wilkins and Patrick list those requirements necessary for

fruitbody production of grassland fungi as;(a), a minimum temperature above 5°C; (b), a maximum temperature below 30°C, and (c), a soil water content above 20% W/W; these requirements are only met at the times of the Spring and Autumn seasonal peaks in fruitbody production.

Grainger. J. (1946) considers soil moisture levels require to lie between 30 and 50% W/W for fruitbody development to take place, increased levels of nitrate which show maxima for April to June and September to November is also considered to promote fruitbody development during these periods. Grainger. M. (1942) considers that T. gambosum "appears with the spring maximum of nitrogen when the lower temperature appears to be sufficient for this species".

Wilkins and Harris (1946) in a consideration of the fungus floras of a Beechwood and a Pinewood report a minimum requirement of (a), a surface litter moisture content above 50% and (b), an arithmetic mean of minimum temperatures above 4°C, as the basic requirements for fruitbody formation which are only met during the respective seasonal peaks in sporophore development. Fluctuation in hydrogen ion concentration of the soils at these sites throughout the year was not determined to be sufficient to influence fungal activity, though individually selective for the fungal floras of each site.

Warcup (1951) reports isolating T. nudum mycelium in alkaline grassland at a depth of 4-5 inches, in an acid grassland the mycelium was isolated at 0-2 inches depth. Mycelium of T. nudum was also found in the litter layer of a pine forest assessed for its basidiomycete flora.

An interesting observation was that the mycelium of Tricholoma melaleucum, Clitocybe rivulosa, Crinipellis stipitarius, Stropharia coronilla and Clavaria argillacea were found associated with the roots and rhizomes of Carex glauca, Carex spp., Festuca ovina, Festuca sp., and Calluna vulgaris respectively. This suggests that grassland fungi may form a beneficial association with the roots of the associated grass species.

L.nuda is recorded as isolated from coniferous forests (Norkrans 1950; Warcup 1951; Rawald 1963; Lundeberg 1970; Lamb 1974) in mixed or deciduous forest locations (Constantin and Matruchot 1898; Votypka 1971); and pastureland soils (Warcup 1951). L.saeva has been isolated from pastureland (Wilkins and Patrick 1940; Norkrans 1950) and deciduous forest (Rawald 1962). C. gambosa has been obtained from meadow (Wilkins and Patrick 1940; Norkrans 1950, 1951; Rawald 1963) and in mixed coniferous and deciduous wood (Rawald 1962).

10:1 Mycorrhizal Associations

Trappe (1962) in a comprehensive review of probable ectotrophic mycorrhizal associations obtained from field observations, reports L.saeva as mycorrhizal with Picea abies, Pinus strobus and Quercus spp.

L.nuda is reported as forming associations with Pinus strobus, Corylus avellana, Populus deltoides and Populus nigra and with Populus nigra and Populus monilifera (Vozzo 1969).

C.gambosa is recorded as mycorrhizal with Prunus spinosa. Trappe makes the point that inability to synthesise mycorrhiza with the fungus and a range of tree spp., in vitro., does not exclude their possible formation in nature.

Lundeberg (1970) in synthesis experiments with T. nudum and Pinus sylvestris failed to establish the presence of any mycorrhizal associations. The presence of T.nudum had a clearly deleterious effect on the seedlings growth, however, the fungus was observed to cause net mineralisation of bound humus nitrogen. Inoculated together with either Boletus subtomentosus or B.variegatus one or the other fungus was dominant in the capsules, the presence B.subtomentosus was considered to reduce the extent of the inhibitory affect of T.nudum on the pine seedlings.

Norkrans (1949) was unable to synthesise mycorrhiza between

seedlings of birch or pine and T.gambosum, although growth of the fungus was reported as good.

McArdle (1932) reports mycorrhiza forming between T.personatum and the conifers Pinus strobus and Picea abies under aseptic conditions, however, Modess (1941) cast aspersions on the validity of this result and considers, together with Melin (1936) and Norkrans (1949), that T.nudum and T.personatum belong to the non-symbiotic purely saprophytic group.

Data from physiological studies on the level of cellulase and laccase activity (Norkrans 1950, Lindeberg 1946, Lundeberg 1970) is considered together with discrepances in protease and pectinase levels with a mycorrhizal fungus for T.nudum (Lundeberg 1970) to place these species firmly in the litter decomposing, saprophytic category.

Isolation of only a restricted range of higher fungi from mycorrhizal roots of a number of pine species performed by Zak and Bryan (1963), Zak and Marx (1964) and Lamb and Richards (1970) indicate that L.nuda, together with a number of other Basidiomycetes forming conspicious fruiting bodies in pine stands, may be obligate rhizoplane inhabitants or are associated with other plants in the forest community. The inability of Lamb and Richards (1970) to synthesise mycorrhizas with this fungus and pine seedlings, indicates that it is at best only a possible minor mycorrhiza former within the forest communities studied.

11:0 CULTIVATION OF FRUITBODIES.

Constantin and Matruchot (1898, 1901), Matruchot (1914), and Constantin (1942) record a number of indoor and outdoor trials concerned with fruiting T. nudum.

Spores germinated and grew readily in tubes on various leaf substrates and also in particular spent tan bark (bark of the Chestnut oak Quercus prinus). This substrate in tubes supported the development of fruitbody primordia.

Mycelium grown in flower-pots under cloches in a warm conservatory on the leaf and bark substrates, produced a "flaky, dense and crusty" growth at the surface. Fruiting took place following two months, primordia which developed were very numerous, exceeding in some cases a 100 per pot, the majority of which, however, atrophied. The largest size attained was approximately stipe height 3 cm, cap diameter 1.5 cm, in a number of cases these fruitbodies are reported to have formed gills and basidia. Fruitbody production on piles of beech leaves within the conservatory was reported to be complete and normal.

Ridge beds were constructed on the floor of a cave from beech, oak, poplar and maple leaves and also spent tan bark and a commercial mushroom growers compost. Initial inoculation was achieved by inserting clumps of leaves permeated with mycelium, obtained from that growing naturally, this was later carried out by transfering mycelium from freshly inoculated beds. Best growth was on beech leaves and spent tan bark. Fruiting took place 7 to 14 months later, generally between 10 and 12 months following inoculation. Stranding was apparent in the region where fruitbodies formed. Fruiting occurred within the cave throughout the year.

Trials outside established the perennial nature of the mycelium and fruiting capacity of the species.

Over the period 1900 to 1914 approximately eleven serial transfers from old to young beds were performed. Following this time Matruchot (1914) records abnormalities in the fruitbodies including loss of colour, a more clitocyboid appearance with a smaller more infundibiform cap bearing decurrent lamellae. A morphological abnormality which occurred throughout the trials was 'gigantism' with occasional individual fruitbodies attaining a verylarge size.

Passecker (1969) obtained fruiting of L.nuda on a partly rotted substrate, consisting of 7 parts scattered fir needles, 1 part alder leaves and 2 parts of horse manure. Inoculum was prepared by subculturing mycelium on malt agar onto a sterile horse manure filled into large containers.

Fruitbodies were obtained two months following inoculation at a room temperature of approximately 12°C and under conditions of low lighting. The fruitbodies produced were of somewhat abnormal appearance, the stipes were abnormally long and somewhat swollen in the manner of blisters, the cap was smaller, paler and infundibiform with decurrent lamellae.

Votýpka (1971) records obtaining primordia at 15°C on sterile Roe deer and horse manure substrates – which had undergone some prior fermentation – at 26 to 29 days after inoculation. The primordia achieved a maximum size of 0.5–2mm at this time, they did not develop further.

In an outside trial Votýpka inoculated a small pile of beech leaves in a sheltered position with mycelium pregrown on Roe deer manure, and obtained single fruitbodies at the end of the first and second months plus a number of smaller fruitbodies and primordia.

Duggar (1905) achieved good growth of T. personatum on leaves, soil, manure and beans by culture in test tubes. Duggar (1929) records obtaining fruitbodies of T. personatum on sterilized leaves also on fermented leaves of maple, beech and birch and on completely fermented stable manure, in the latter case obtaining well formed mushrooms two months following inoculation. Some confusion exists, however, taxonomically, from the former use of the interchangeable names T. nudum and T. personatum, from a description of fruitbody form and to references in the literature, therefore, it is possible that the former species was in fact cultivated.

Zadražil, Schneidereit, Pump and Kusters (1973) report T. nudum mycelium grew well and was resistant to infection. Details of cultivation were not given, but they report obtaining fruitbodies at a temperature of 10°C, 270 days following inoculation.

Voltz (1966) attempted mushroom house cultivation of L. nuda using a commercial compost as substrate. He reports dense, purple hyphal growth produced over the compost surface and also a mycelial mat over the surface of the casing soil, however, no fruitbody initials developed.

With the exception of Duggar's account no description of fruiting in culture of either Lepista saeva or Calocybe gambosa are available.

12:0 SUMMARY.

Optimum growth is indicated for the three species Lepista nuda, Lepista saeva and Calocybe gambosa at 25°C and 20°C for the latter species. Hydrogen ion concentrations at pH 5.0 to 6.0 are recorded as optimal.

Isolates of Lepista nuda appear to show considerable diversity in their ability to grow on a wide range of carbon and nitrogen sources. Poor growth on certain of the carbon sources may be caused by a long lag period resulting from insufficient energy to promote inducible enzyme systems required for the utilisation of the substrate. This can be circumvented by addition of 'start' glucose or may not arise where the fungus can make sufficient initial growth on other substances in the media (Lamb 1974). This may equally be the case for utilisation of nitrogen sources.

There is evidence that all three species can breakdown cellulose and lignin, although the extent of these individual enzymic capacities has not been clearly defined.

L.nuda and C.gambosa have been demonstrated to show a requirement for thiamine, this has been determined to be due to an inability to synthesise the pyrimidine molecule (Norkrans 1950). The results of Rawalds (1963) investigations, however, leave the overall vitamin requirements for the three species in an uncertain position.

Asparagine, glutamine and their corresponding keto-acids and half amides appear to stimulate growth of L.nuda and C.gambosa. There is evidence that C.gambosa may have certain deficiencies in peptide synthesis (Norkrans 1953).

All mycorrhizal synthesis experiments with isolates of L.nuda,

L. saeva and C. gambosa which proved negative were undertaken with pine seedling species, except for one instance with birch, however, these fungi are also associated with deciduous woods (possibly to a greater extent in the case of the two latter species) therefore synthesis experiments with a range of hardwood tree species requires to be undertaken before the ability to form a mycorrhizal association can be dismissed. In the absence of any mycorrhizal formation, an intimate association with plant roots in the rhizosphere of soft and hardwood tree communities and possibly grassland communities is probably important in a consideration of the ecology of these species.

Isolates of L. nuda used in the physiological studies were all obtained from coniferous locations where mycorrhizal association has been generally suspected; this fungus occupies a wide range of habitats including deciduous leaf litter and rich soils, where the fungus would be better able to maintain an independant existance. Therefore, to some extent previous investigators may have presented a one-sided representation of its physiology.

Little information on factors inducing fruit-body formation within these species is available, the fruiting of these species in spring and winter months distinct from the main autumn peak in sporophore numbers indicates individual requirements for conditions conducive to sporophore formation. The presence of Calocybe gambosa in the spring may be due to slow mycelial growth by this species, particularly at the low temperatures prevailing during the winter months, the high surface soil temperatures of spring being required to produce strong growth. It may also be that fruit-body production by this species occurs in response to a rapid rise in surface soil temperature during spring. Although such temperatures are unlikely to be directly inhibitory to growth, the fruiting response may be due to drying of the surface soil or the development of a high level of saprophytic competition in the surface soils or leaf-litter.

MATERIALS AND METHODS

MATERIALS AND METHODS

1:0 Fruitbody Characteristics.

Fruitbodies of L.nuda and L.saeva show considerable variation in type of habitat, proportions of cap and stipe and degree of coloration, with colour fading in both in older specimens, some collections of L.nuda turning distinct russet-brown when allowed to dry out. These variations in fruitbody form and location have led to the provision by a number of taxonomists of a variety of subspecies within each group (Ramsbottom. 1951; Lange J. 1933); descriptions are more consistant for C.gambosa. The taxonomic critera of these three species has been thoroughly investigated by Singer (1962). A more concise approach to nomenclature has been recently adapted and taxonomic confines of each species more clearly defined. (Dennis, Orton and Hora, 1960).

In general the isolates collected agreed well with the descriptions of Ramsbottom (1951), Wakefield and Dennis (1950), and Watling (1973).

Variation was particularly evident in collections of L.nuda (approx.,14 collections, 7 not recorded, made by author) which is considered to be a highly adaptable fungus occupying a wide range of habitats including positions in composts, leaf litters, rich soils and more exceptionally open permanent pasture land. Variation in height (3 cm to approx. 20 cm), girth and strength of purple coloration was considerable; certain isolates of L.saeva were particularly large heavy specimens. Fruitbody descriptions of Lange and Hora (1965;colour plates from J.Lange,Flora Agaricina Danica,1864-1941) for L.nuda and L.saeva were generally confusing, particularly L.nuda where only one isolate possessed a distinct red-brown cap cuticle, L.saeva possessing only blue veins from the base of the stipe fading towards the stipe apex which together with the cap lacks any lilaceous coloration. Specimens of L.nuda possess a distinct aromatic smell absent in L.saeva.

Generally descriptions of C.gambosa were similar and agreed well with specimens collected. This genus contains several tropical species (Singer,1962; Purkayastha and Chandra,1974). Its members typically possess carminophilic granules in the basidia.

TABLE: 1:0 ORIGIN OF ISOLATES

ISOLATES	DATE OF ISOLATION	LOCALITY	HABITAT	SOURCE
Lepista nuda				
1	2/72	Reading, Oxfordshire		Dr. Worgan
247.69	30/9/68	St.Hubert. S. Netherlands		C.B.S
300.58	1958	Weimar, Germany.		C.B.S
493.48	1948	Sacávem, Portugal.		C.B.S
5	10/73	Stourbridge, Warwickshire.	Grass Cuttings	
6	10/73	Stagborough Hill, Worcestershire	Permanent Pasture	
7	10/73	Pipers Hill Warwickshire	Mixed Wood	
8	10/73	Earlswood Lakes, Warwickshire.	Mixed Wood	
9	11/73	Sheffield, Yorks.	Old Flower Bed	
10	11/73	Orchard Brae, Scotland.	Under Hawthorne	Dr. Watling 1
11	1/72	Royal Botanic Gdns., Scotland.	Amongst Cinders by Gate	Dr. Watling 2
12	11/74	University Campus Birmingham.	Recently Turfed Lawn	
13	10/74	Chancet Wood, Yorks.	Mixed Wood Under Privet	
Lepista saeva				
1	1/73	Warwick, Warwickshire.	Pasture	
291.61	10/9/60	Bohem, Germany.		C.B.S
248.69	27/11/67	Longhill, Leicester.		C.B.S
355.33	1933	Germany.		C.B.S
5	10/73	Stourbridge, Warwicks.	Grass Lawn	
6A	10/73	Stagborough Hill, Worcestershire.	Mixed Wood	
6B	10/73	Stagborough Hill.	Pasture	
7	11/74	Burton-on-Trent.	Pasture	
8	10/74	Mays Wood, Warwicks.	Pasture.	

TABLE: 1:0 (cont.,)

ISOLATES	DATE OF ISOLATION	LOCALITY	HABITAT	SOURCE
Calocybe gambosa				
1	8/5/73	Great Alne, Warwickshire.		
2	5/73	Stoke Prior, Warwickshire.	Bordering Mixed Wood	
3	5/74	Moreton Bagot, Warwickshire.	Mixed Wood & Roadside Verge bordering mixed Wood.	
4	5/74	Droitw ich, Worcestershire	Roadside verge, bordering mixed Wood.	
5	5/74	Thornhill Wood, Little Alne, Warwickshire.	Grass bank bordering mixed Wood.	

1.	Watling Herbarium No. 10123.	C.B.S.-Central bureau voor
2.	Watling Herbarium No. 8896	Schimmel Cultures, Baarn, Netherlands.

P L A T E 1.

Sporophores of Lepista nuda obtained growing wild.

The older flanking specimens from the same collection have turned reddish-brown (russet) upon drying (Isolate No.7).

The central specimen indicates the original light lilaceous colouration (a pinkish overtone has been introduced in reproduction of the photograph). (Isolate No.8.).

SCALE 2 cm.

P L A T E 2.

Sporophore of Lepista nuda of stout proportions with small deep purple cap and thick lighter coloured stipe. Growing within a deep pile of beech leaves.

SCALE 1 cm.

P L A T E 3.

Very robust sporophores of Lepista nuda with the typical 'tricholomatoid' inverted cap. Virtual absence of colouration in the gills and speckled lilaceous stipe Growing amongst mixed deciduous leaves at the bottom of a garden.

SCALE 1 cm.

P L A T E 4.

Lepista nuda sporophores of relatively delicate proportions from compost pile. Purple colour more strongly developed in the gills, light colour on the stipe.

SCALE 1 cm.

P L A T E 5.

Robust sporophore of Lepista saeva. Purple colour developed in veins at the base of the stipe fades rapidly after collection.
(Isolate 5.)

SCALE 1 cm.

P L A T E 6.

Sporophores of Calocybe gambosa. Obtained from well-rotted leaf humus in roadside verge.
(Isolate No.4).
Cap surface with reddish tints.

P L A T E 7.

Sporophores of Calocybe gambosa. Obtained from grassy bank. Distortion of cap by grass roots during emergence producing typical 'gamboid' (hoofed) appearance.
(Isolate No.5).

SCALE WIDTH OF COIN 2.8 cm.

The cleft appearance of the fruitbody is a distinct feature of this species.

Species in the genus Lepista typically possess a sordid to cream pink spore print, the spores have a thin, hyaline finely roughened spore wall, characters which have formed the basis for its delimitation from the closely allied genera Tricholoma and Clitocybe.

C.gambosa is a vernal species, L.nuda and L.saeva fruiting in late autumn and winter, the former (in Scotland) fruiting in quantity after the first frosts (Watling, personal communication). The time and duration of fruiting is highly dependant on the temperature and rainfall of the individual season, generally C.gambosa was collected in late May and did not appear as early as 23rd. April (St. Georges Day), fruitbodies of L.nuda and L. saeva were collected as late as February in the mild winters of 1974 and 1975.

1:1 Taxonomy.

Classification according to Dennis, Orton and Hora (1960) 'The New Check List'. With the exception that Calocybe gambosa is used in preference to Tricholoma gambosum.

Lepista nuda (Bull.ex Fr.) Cooke		(the 'wood blewit')
Syn:	Tricholoma nudum	Bull.ex Fr.
	Rhodopaxillus nudus	(Fr.ex Bull.) Maire
Lepista saeva (Fr.)	Orton	(the 'pasture blewit')
Syn:	Tricholoma personatum.	Fr.
Agaricus saevus Fr.	Lepista personatum.	(Fr.ex Fr.) Cooke
	Rhodopaxillus saevus	(Gill.) Maire
Calocybe gambosa (Fr.) Sing.		(the 'St Georges Mushroom').
Syn:	Tricholoma gambosum	(Fr.) Kummer.
	Lyophyllum gambosum	(Fr.) Sing.
	Tricholma graveolens	(Pers ex Fr.) Kummer
	Tricholoma albellum	(DC.ex Merat) Kummer
	Tricholoma georgii	(L. ex Fr.) Quél.
	Calocybe georgii	(L. ex Fr.) Kühner
	Lyophyllum georgii	(L. ex Fr.) Kühner & Romagn.
	Agaricus prunulus.	Vitt. non Scupoli Fr.

2:0 CULTURE OF ISOLATES.

Tissue blocks were taken from pileus tissue at the general apex of the stipe following the aseptic procedures outlined by Watling (1971) and half submerged in fresh 2% malt extract agar (Pantidou 1961). Hyphae generally grew well from the aerial portion of the tissue block. Where the material was not fresh (as in the case of L. saeva which tended to take-up water in the field) generally aureomycin 2-6 mg/L (or occasionally streptomycin or penicillin) was incorporated into the agar. With C. gambosa which was initially slow growing, benomyl (Taylor 1971) was added at 5mg/L to suppress possible phyco-and ascomycete contaminants.

Cultures were maintained on 2% malt agar slopes in 20 ml capacity McCartney bottles at 4°C.

3:0 STUDIES IN PETRI-PLATES.

9cm sterile disposable polystyrene Petri-plates were employed. Approximately 20 ml 2% Boots malt extract agar was used for routine culture work. Exact 20 ml quantities of media were introduced for the experimental studies.

Inoculation, at the centre of each plate was carried out with a No. 3 corkborer (6mm dia.,) mycelium agar plugs were taken from 1 cm within the periphery of 3-4 week old colonies. Excess staled agar was cut from beneath the mycelial disc to enable rapid growth down on to the new medium and minimise carry-over of staling products.

Temperature studies were conducted as an assessment of the rate of mycelial extension for 4, 3 and 1 isolate of L. nuda, L. saeva and C. gambosa respectively, maintained in incubators or fridges at the stated temperature. Colony diameters at right angles were measured and averaged for two replicates at each temperature.

Assessment of p-diphenol oxidase activity on gallic and tannic acid media was made for a range of L. nuda(13), L. saeva(7) and

and C.gambosa (2 isolates) following the procedures outlined by Davidson, Campbell and Blandell (1938) and Lindeberg (1946). Gallic and tannic acids were millipore filtered (0.44/mm membrane pore dia.,) and added at concs., of 850 mg/L and 500 mg/L to glucose-asparagine (to provide a clear medium for faint reactions) and 2% malt extract agar media respectively. 6 mm and 18 mm mycelium agar inocula were employed, one and two inocula per plate for gallic and tannic acid media respectively, plates in replicates of three.

Growth and gross colony morphology for one isolate each of L.nuda and L.saeva and C.gambosa was assessed on glucose-asparagine basal agar medium with the inclusion of a range of lower aliphatic acids and fatty acids for duplicate plates at concentrations of 0.5, 1 and 5 g/L. Methods of sterilisation and addition followed those for the procedures given in a later section on stationary culture in flasks, the organic acids and lipids being added and dispersed in the cooled mediumjust prior to pouring.

Growth and clearing of 11 isolates of L.nuda, 6 isolates of L.saeva and 2 isolates of C.gambosa was determined on a modified Norkrans Cellulose medium (appendix) which contained 1% 72 hr ball-milled cellulose finely dispersed to give a white opaque medium (Eggins and Pugh, 1962). Growth and cellulolytic ability was measured as mycelial extension and development of clearing along 2 diameters at right angles for duplicate plates.

All isolates were assessed for growth on malt agar following isolation, leading to selection of those strains giving best growth for more extended studies.

Three isolates of L.nuda, 2 of L.saeva and 2 of C.gambosa were further grown in triplicate on potato dextrose, chlamydospore and Eggins and Pugh Cellulose media,to check for stranding and rhizomorph formation as reported by Voltz (1966) for his strain of L.nuda on chlamydospore agar, and observed to be formed by certain isolates during the course of studies on cellulose media.

3:1 Studies with divided Petri-plates.

Sterile disposable polystyrene Petri-plates with a single vertical partition extending to approximately two thirds the height of the Petri-plate base were employed.

The principle consisted of establishing a vegetative and reproductive substrate in the two halves of the plate (Hume and Hayes 1972). Agars were added in volumes of 20 ml per side, the former was always 2% Boots malt agar solidified with 1.5% Oxoid agar No. 3. The vegetative medium was the experimental substrate, this consisted of either (a) 1.5% water-agar, (b) Skermans carbon free medium agar (Skerman 1959), (c) Soil extract agar, (d) a sterile peat/chalk mix (approx., proportions chalk/peat/water 1:2.3:5 $^{w}/_{w}$) containing 25% $^{v}/_{v}$ granulated activated charcoal (Long 1970), (e) a sterile water agar/granulated actived charcoal mix (in proportions, 3:1 $^{v}/_{v}$, total volume 20 ml). These media were employed for three isolates of each of the species L.nuda (7, 8 & 13) L.saeva (248.69, 5 &6B) and C.gambosa (1, 3 & 5) in duplicate plates.

Inoculation was achieved by two equally spaced 6 mm agar inocula on the malt agar medium. Incubation at 25°C continued until the fungus had overgrown approx., one third of the experimental substrate (3-4 weeks), at this stage the plates were transfered to cellar (16°C) or room (20°C) temperature. Where mixed bacterial 6 mm water agar plugs were employed these were placed in pairs on the water agar (medium (a)), equally spaced, opposing the mycelial inoculation plugs.

For the incubation at the lower temperature a dry enviroment was sought to reduce the relative humidity and condensation of water vapour within the Petri-plate. This phase of incubation was extended upto two months duration.

4:0 ISOLATION OF BACTERIA AND MIXED INOCULATION PROCEDURES.

Ninety-nine bacteria isolates were recovered from dilution plates made on nutrient and soil extract agars, of five soils obtained from

Coleshill, Birmingham (permanent pasture supporting a fairy-ring of C.gambosa) Moreton Bagot, Stagborough Hill, Droitwich and Evington Meadows. The isolates were characterised by morphology though not identified to the generic level (with the exception of 8 fluorescent pseudomonad sp., determined on King's Medium B). Three strains of L.nuda (5, 7 & 8), L.saeva (248.69, 5 & 6B) and C.gambosa (1, 3 & 5) were assessed for growth inhibition caused by these isolates, by radially streaking the isolates out from a central c. 3 cm fungal colony on malt extract agar.

The effects of cell-free filtrates of mixed bacterial flora from the five individual soils plus a sixth 'casing-soil' sample from a currently cropping mushroom bed of Agaricus bisporus was assessed by millipore-filtration of the combined bacterial isolates from each soil, grown-up in 100 ml quantities of soil extract medium for 3 days as a shake flask culture, as 2, 4 & 6 ml additions of the filtrate to 20 ml of soil extract agar. Growth was assessed by single isolates of L.nuda (247.69), L.saeva (248.69) and C.gambosa (1) from 1.8 cm mycelial malt agar inocula placed centrally in the Petri-plate. The plates were in triplicate with soil extract medium as control.

4:1 Preparation of Soil Extract Agar.

The soil extract medium consisted of: Soil extract stock soln., 100 ml, KH_2PO_4 0.5g., Glucose 1.0g Agar 15g., Tap water 900 ml.

The stock soln., of soil extract was prepared by steaming 1kg of soil from Evington Meadows, Leicestershire, (Clay loam) with 1 litre of tap-water in an autoclave for 30 mins., This was mixed and dispersed to two 1 litre measuring cylinders and 2.5g calcium carbonate added to each and allowed to stand for one hour. The supernatant liquid was poured-off and was repeatly filtered through a double layer of Whatman No. 1. filter paper until clear. This was then autoclaved at 15 p.s.i. for 20 minutes and maintained sterile by aseptic addition to the agar medium. The stock soln., was held in the cold store at $4^{\circ}C$ for further use.

5:0 GROWTH IN FLASKS.

6 mm inocula obtained and treated as given in the previous section were placed on fresh 2% malt agar plates and incubated at 25°C for 24-48 hrs. to enable the formation of aerial hyphae, which greatly facilitated floating the inocula on the liquid medium (Norkrans 1950). Selection of only those inocula with abundant aerial hyphae also ensured uniformity of inocula throughout the experiment and reduced the initial lag period of growth.

100 ml wide-mouth Pyrex Erlenmeyer flasks were used throughout, stoppered with non-absorbent bleached cotton-wool and containing 25ml of the synthetic liquid medium. Certain of the experiments were incubated in a standard gravity convection incubator at 24°C ± 1°C, the larger experimental systems were incubated at room temperature (22°C±2°C). Generally flasks were incubated for 28 days occasionally shorter or longer, as indicated, and once dry-weights were recorded at 7 day intervals. With the exception of C/N ratio, pH and leaf extract studies (duplicate flasks), all experiments were conducted with replicates of 3 flasks per treatment. Flasks and associated glass-ware were cleaned in Pyroneg (Diversey Products) -a phosphate based detergent - and rinsed finally with several changes of deionised water.

For assessment of growth, mycelial mats were drained on a fine wire/ mesh supported over a Buchner funnel and flask maintained under suction. This enabled the combined culture fluids to be collected and pooled, the mesh was then transferred over a sink and the mats washed with deionized water contained in a wash bottle. The drained mycelial mats were then transferred to previously dried and tared 7 cm. dia., filter-paper discs or in later experiments tared squares of aluminium foil. The mats were oven dried at 65°C to constant weight and weighed to the nearest tenth of a milligram on a Mettler balance. The use of aluminium foil enabled the dried mycelium to be readily removed for analysis and also further minimised errors in the final weighing by eliminating uptake of water which is otherwise absorbed by the dried filter-paper disc.

Growth was assessed where ball-milled cellulose and calcium oxalate were used as carbon sources, by measurement of total Kjeldahl N (Jacobs 1965), and conversion to dry-weight by comparison with dry-weight and nitrogen values of mycelial mats formed by growth with glucose as carbon source.

The basal medium used for stationary culture in flasks was a modified Norkrans glucose-asparagine medium adjusted to pH 6.0 with appropriate quantities of m/15 pot. dihydrogen orthophosphate and disodium hydrogen orthophosphate as indicated in a later section dealing with pH. This resulted in a phosphate concentration at this pH of 0.00614 Molar. Initial experiments as indicated contained only 1g asparagine/L. The adaption of ferric citrate as iron source instead of ferric chloride was found to considerably improve growth in initial trial experiments. The level of thiamine was increased from 50 to 200μg to allow sufficient quantity for the greatly increased yields obtained in these experiments to those of Norkrans (1950,'53), and a biotin addition was made as certain strains indicated at least a partial requirement for this substance from the vitamin studies.

The basal medium was composed of: Glucose 20g., Asparagine 2g., M/15 kH_2PO_4 131.5 ml and M/15 Na_2HPO_4 18.5 ml to give pH 6.0., $MgSO_4.7H_2O$ 0.5g., NaCl, 0.1g., Ferric citrate 5mg (Iron stock soln. contains 1g Ferric citrate ($\equiv$ 0.17gFe) and 0.64g Citric acid per 100 ml) Zn $SO4.7H_2O$ 4.4mg., $CaCl_2.2H_2O$ 0.1g., Thiamine 200μg Biotin 5μg Distilled water to 1 litre. pH 6.0 best approximated to the optimum pH for strains of L.nuda, L.saeva and C.gambosa from pH studies.

Sterilisation of carbon and nitrogen sources wherever possible was carried out by millipore filtration using a cellulose acetate filter of 0.44μm pore size. Where media required to be autoclaved this was achieved at 15 p.s.i. for 5 minutes to keep time in the autoclave to a minimum. Generally individual media components were sterilised in 200 ml medical flats in a small stainless steel autoclave with a rapid cycle time. Sterilised materials were pipetted into flasks, tubes and bottles using

standard aseptic procedures. Millipore sterilisation and individual autoclaving procedures were essential to prevent decomposition of medium components, the production of inhibitory substances in the media (Cochrane 1958), and the generally concurrent changes in pH and exhaustion of buffer capacity.

All chemicals employed in these studies were of 'Analar' or 'puriss' grade.

6:0 GROWTH STUDIES. pH ADJUSTMENT AND STERILISATION.

6:1 Determination of Optimum pH.

Growth over the pH range 2 to 8 was assessed for 3 strains each of L.nuda and L.saeva plus a single strain of C.gambosa utilising the glucose-asparagine synthetic medium containing 1g asparagine/L. Incubation was at 25°C for 21 days. Individual quantities of media were made-up and adjusted to unit values within the stated pH range by combination of the Sorensen phosphate buffers m/15 KH_2PO_4 + N/10 HCI, M/15 kH_2PO_4 +M/15 Na_2HPO_4 according to the table of Lindeberg (1939). Actual pH values for each quantity were determined by a combined glass pH electrode and meter, these were then sterilised by millpore filtration and 25 ml quantities aseptically pipetted into oven sterilised Erlenmeyer flasks.

Hydrogen ion concentration of the pooled culture solutions for the 3 replicates was taken following growth of the isolates for 3 weeks to determine pH shift. This method was found to be the only solution to circumvent the considerable alteration to the media or components of it which occur with autoclaving, and the concurrent shift in pH which is particularly marked in the alkaline range.

6:2 Carbon: Nitrogen ratio

Assessment of the optimum C/N ratio was conducted for a single strain of L.nuda and L.saeva. Concentrations of asparagine were chosen at 0.1 (0.1864 gN/L) and 0.3 per cent (0.5592 gN/L) at carbon:

nitrogen ratios for spacings of 3 units over the range 9 to 28:1, by altering the level of glucose addition in each series. Duplicate flasks for each strain at the individual ratios were harvested every 7 days over a 28 day period and the dry-weights and pH of the pooled culture fluids recorded.

Values for mycelial dry-weights and pH of the pooled culture fluid following 28 days incubation were obtained for single isolates of L.nuda, L.saeva and C.gambosa cultured in four series of C:N ratios at individual nitrogen concentrations of 0.00932, 0.01864, 0.03728 and 0.05592 per cent. C:N ratios within each series were achieved by alteration of the carbon level. Carbon and nitrogen concentrations were obtained by manipulation of glucose and asparagine concentrations within the standard basal liquid medium. Values are for duplicate flasks.

6:3 Vitamin requirement.

The vitamin requirement was determined for 2 strains each of L.nuda, L.saeva and C. gambosa utilising the basal medium plus vitamin addition. Asparagine added at 1g/L was purified by treatment with finely divided Norit activated charcoal according to Yusef(1953) in an attempt to remove vitamin sources.

The vitamin sources and yeast extract were sterilised by millipore filtration. The growth requirement was assessed by removal of individual vitamin sources from the complete vitamin mix which contained inositol, choline chloride and lipoic acid as additional substances in an attempt/ to simulate the composition of yeast extract.

All vitamin sources were added at a concentration of 200μg/l with the exception of biotin (5μg), choline chloride (200mg) and lipoic acid (20 mg). P-aminobenzoic acid is part of the folic acid moity. Yeast extract was added at 0.4 g/L. The glucose and asparagine were millipore sterilised and added in appropriate concentration to the prior autoclaved basal salt soln., made-up to the requisite concentration.

6:4 Utilisation of Carbon Sources.

Single strains of L. nuda, L. saeva and C. gambosa were cultured on 32 single carbon sources and 11 combinations of carbon sources at a concentration of 8g C/L: raffinose, starch, inulin and dextrin additions were corrected for loss on drying. Asparagine was added as nitrogen source at 2g/L. The nitrogen source for C. gambosa consisted of 19 amino-acids added in equal quantities of nitrogen as given for the amino -acid mix in the following section. This was added to and autoclaved with the basal salt soln., plus vitamins at 15 p.s.i. for 5 minutes.

All soluble carbon sources were pH adjusted to 6.0, millipore sterilised and added to the prior autoclaved basal salt solution. Insoluble carbon sources were autoclaved with the basal salt soln., for 5 minutes at 15 p.s.i. Asparagine was filter sterilised and added separately. Autoclaving resulted in some hydrolysis of the inulin to fructose and possible partial hydrolysis of starch and pectin to their constituent sugars. Flasks containing insoluble carbon sources required pH adjustment to 6.0 following autoclaving. Flasks were incubated at room temperature ($22^{\circ} \pm 2^{\circ}C$) for 28 days (L. nuda and L. saeva) and 32 days (C. gambosa).

6:5 Utilisation of Nitrogen Sources.

Growth was determined for two isolates each of L. nuda, L. saeva and C. gambosa on a range of complex organic nitrogen sources, ammonium, nitrate and nitrite nitrogen. Nitrogen souces were added at a concentration of 0.3728 gN/L equivalent to 2g asparagine/L. All nitrogen sources were pH adjusted to 6.0 then individually filter sterilised and added separately together with millipore filtered glucose to the prior autoclaved basal salt and vitamin solution.

Growth of single strains of L. nuda, L. saeva and C. gambosa was assessed for 19 amino-acids as sole nitrogen sources plus the amides asparagine and glutamine. Growth in a synthetic amino-acid mix which contained the 19 amino-acids in equal proportions of nitrogen and on casein hydrolysate was also determined. All nitrogen sources individual and combined were added at 0.3728g N/L except as indicated, and pH adjusted

to 6.0 prior to sterilisation. Sterilisation, due to limited quantities available of many of the amino-acids, was achieved by individual autoclaving at 15 p.s.i. for 5 minutes.

Amino-acids were generally added as the L-isomeric form, racemic forms of alanine, methionine, valine and phenylalanine were added at double the concentration of nitrogen on the assumption that only the L-form is utilised (Reusser, Spencer and Sallans, 1958 b). L-cystine and L-tyrosine were largely insoluble and DL-β-phenylalanine partly soluble at pH 6.0.

6:6 Effect of Fatty and Organic acids on growth.

The effect of a range of saturated and unsaturated fatty acids and organic acids on growth of single strains of L.nuda, L.saeva and C.gambosa was determined in the synthetic glucose-asparagine medium containing 1g asparagine per litre. Linoleic, oleic, palmitic and stearic acids (100 and 500 mg/L) were diluted indiethyl ether and added to the glucose-salts soln., in flasks,prior to autoclaving. Acetaldehyde and acetic acid (50 and 100 mg/L) which effect the cellulose acetate membrane filter if filter sterilised were also added directly to the flasks prior to autoclaving.

Ethanol (100 and 500 mg/L) propionic, iso-valeric and buteric acids (50 and 100 mg/L) were diluted, millipore filtered and pipetted into the autoclaved, glucose-salts medium. Asparagine was added separately following filter sterilisation. All treatments were in replicates of three. The flasks were incubated at 24°C for 28 days. Following autoclaving (during which the diethyl ether was dispelled) the fatty acids tended to form droplets on the surface of the medium.

6:7 Effect of Ash and Metal ions on growth.

The importance of various elements in the basal salt soln., was approximately assessed for two strains of L.nuda and L.saeva.

Additions were made to a basal soln., which contained: KH_2PO_4 and $Na_2H\ PO_4$, KCL , $MgSO_4 \cdot 7H_2O$ and ferric citrate plus thiamine and biotin vitamin additions at concentrations similar to those for the synthetic glucose-asparagine medium, potassium chloride replacing sodium chloride. Ash additions were made at 0.4 g/L. The synthetic mineral soln., was formulated as a synthetic yeast extract ash and contained per litre: $CaCl_2 \cdot 2H_2O$ 0.5 mg , $(NH_4)_6\ MO_7\ O_{24}$ 0.23 mg , $CuSO_4 \cdot 7H_2O$ 0.5 mg , $MnSO4 \cdot 7H_2O$ 0.49mg , $CuSO_4 \cdot 7H_2O$ 0.59 mg , the amounts and composition being similar to that given by Coscarelli and Pramer (1962) with alterations to allow for a smaller quantity of nutrient medium per flask and the use of sulphates not oxides of manganese and zinc.

The leaf and manure preparations were obtained as given in a later section. Malt extract and yeast extract werepowdered commercial preparations. The ash was obtained by ignition at 450°C for 18 hours in a muffle furnace. The lower temperature prevents the destruction of potassium. Ash preparations were solubilised in small quantities of conc.HCL (Coscarelli and Pramer, 1962). Zinc oxide and zinc oxide plus calcium chloride additions to the basal salt soln., were made at concentrations given for the synthetic mineral soln.. This treatment was also extended to two strains of C.gambosa where the nitrogen source consisted of DL-valine, DL-methionine, L-glutamic, L-aspartic acid, L-β-phenylalanine,

L-serine, L-tryptophan and L-cystine at equal quantities of nitrogen totalling 0.3728 g N/L. This combination was formulated as a substitute for the amino-acid mix used in nitrogen source studies. However, very little growth took place and the results are not included, it is concluded that this combination of amino-acids is very inhibitory to growth of C.gambosa (this was also the case for certain amino-acid formulations assessed by Norkrans, 1950).

6:8 Growth responce to leaf and manure extracts.

The growth responses of single isolates of L.nuda, L.saeva and C.gambosa were determined for the addition of a range of 3 animal manures and leaf extracts from 5 deciduous and 4 coniferous trees to the basal glucose-asparagine medium containing 1g asparagine per litre. The leaf and manure extracts were added at concentrations of 0.5, 1 and 3 mg dry matter per ml according to Melin (1946). Figures were obtained for the mean of 2 replicates at each concentration, flasks incubated at 24°C and harvested following 21 days of growth.

Freshly fallen leaves were collected in autumn, pure leaf collections being made for each tree species. The leaves were rinsed and blended to fragment them to small size. The leaf macerates together with the animal manures were 72 hr ball-milled in a 4% soln.,. Dry-weights were taken for 10 ml samples following ball-milling and the extracts finally sterilised by autoclaving at 15 p.s.i. for 1 hour. This treatment enabled the extracts to be readily pipetted into flasks and the finely divided form provided availability of any growth stimulatory substances that existed in the solid and liquid components.

7:0 CLEARING IN CELLULOSE COLUMNS.

The celluloytic ability of 9 isolates of L.nuda, 7 isolates of L.saeva and 2 isolates of C.gambosa was assessed using the cellulose column technique of Rautela and Cowling (1966).

This was basically modified by using the 72 hr. ball-milled

cellulose medium of Eggins and Pugh (1962). The phosphate buffer combination at pH 6.0, as used in the basal medium, replaced the potassium dihydrogen orthophosphate of the cellulose medium. The technique involves measuring the rate of clearing down a cellulose column beneath the fungus mat. The clearing is clearly visible against the milk-white opaque agar.

Cellulose concentrations of 0.25 and 1% were used, 15 ml of medium dispensed into 20 ml capacity screw-cap McCartney bottles. It was necessary to agitate the medium (cooled to 45°C) thoroughly, then plunge the tubes into ice, to ensure the particles of cellulose were uniformly distributed throughout the final cellulose agar column. The use of screw-caps, partly loosened, permitted the tubes to be incubated at room temperature over two months without drying and shrinkage of the agar; this is an inherent problem where the standard boiling-tubes of Rautela and Cowling plugged with cotton-wool are used. The tubes for each strain were in replicates of 5.

8:0 GROWTH IN TUBES.

Growth rates on solid substrates were assessed using glass tubes of 10" length and $1\frac{1}{4}$" width, plugged at both ends with bleached non-absorbent cotton-wool. This arrangement enabled the tubes to be more evenly packed throughout, than would be the case with test-tubes and furthermore allowed for free gaseous exchange along the length of the column.

Growth on a commercial 'short-straw' mushroom compost (approx. 2.3g% N and 36% dry-matter) was recorded with 130g compost packed as evenly as possible into the tubes, for 9 isolates of L.nuda, 5 isolates of L.saeva and 2 isolates of C.gambosa.

The tubes were autoclaved for 1 hour at 10 p.s.i. on 3 separate days, inoculated with a 1.8 cm agar-mycelium disc, and capped with approx., 1" of a sterile thoroughly moistened peat-chalk mix to prevent drying-out of the agar - this in effect acted as a standard casing layer in which development of strands and possible initials was most obvious.

Overall growth-rate was determined in mm/day, together with an empirical assessment of mycelium density and development of strands for 3 replicates of each strain.

8:1 Growth in flasks.

Growth in 250 ml wide-mouth Erlenmeyer flasks containing 60g autoclaved compost (treated similarly to that given for tubes), plus an addition of 8g autoclaved peat-chalk casing following spawn-run, was assessed for 5 isolates each of L.nuda and L.saeva. Theflasks were inoculated with 1.8 cm mycelium-agar discs and spawn-run at 25°C for 1 month. Extent of stranding and indications of possible formation of initials in the casing were recorded as an extension of the results from growth in tubes.

9:0 FRUITBODY DEVELOPMENT ON AGAR.

Cultures of 3 isolates of L.nuda, L.saeva and C.gambosa were assessed for fruitbody formation on a natural agar medium at 12°C with a Circadian light cycle of 9 hrs light 15 hrs dark supplied by 'daylight' fluorescent lamps.

100 ml quantities of 2% malt extract agar supplemented with 1g/L yeast extract was dispensed into 250 mlwide-mouth Erlenmeyer flasks and the fungus cultures inoculated centrally with 6 mm inocula, 5 replicates per strain, and allowed to overgrow the agar surface for 3 weeks at 25°C. The appearance of stranding, possible primordia and fruitbodies was noted over a period of 5 months.

9:1 Fruitbody development on solid substrates.

Six 5 kg quantities of commercial mushroom compost were spawned at a 50:1% ratio with 3 strains of L.nuda and L.saeva and each transferred to 4 containers (dimensions: 6" square, slightly tapering to 7" depth) at 1.25 kg per container. 3 further 10 kg amounts of compost in plastic bags were spawned with the isolates of L.saeva.

A prior prepared rye grain spawn of these 6 isolates was used as inoculum. Spawn-run continued for 32 days at *c.* 25°C following which the compost was fully permeated with mycelium except in the bag containing *L. saeva* 248.69, which was discarded. The spawn-run compost in the bags was transferred to six containers, 1.67 kg amounts to each.

The containers were cased with *c.* 1½" layers over the separate halves of the spawn-run compost surface with 4 combinations: loam soil and standard peat chalk casing, pear and oak leaves, beech and birch leaves, sitka spruce and larch leaves, a treatment for each container. These materials had received a prior fumigation with methyl bromide for 3 days. The 6 additional containers for the two isolates of *L. saeva* were equally subdivided and cased entirely with commercial peat-chalk casing or loam soil, these containers did not receive any further casing treatment. Containers were initially covered with a layer of plastic, including during spawn-run, to maintain high humidity and moist conditions in the surface layers, and incubated at approximately 16°C.

Following 40 days the containers received a 2nd casing treatment; at this stage the *L. nuda* isolates had produced a very strong growth through the leaf and soil layers with formation of a dense surface lilaceous layer. Growth of *L. saeva* isolates was generally weak through the leaf layers though considerably stronger through the soil. The second casing consisted of an additional layer of clay loam and peat-chalk separately positioned on the four quarters of the mycelium surface, checkerboard fashion, laid thin centrally increasing in thickness towards the corners of the container. Incubation continued at approximately 16°C under conditions of intermittent dim fluorescent lighting for a further 60 days, with regular watering to maintain the casing material moist.

Records were made of those isolates fruiting and individual containers dissected to determine the number of fruitbodies forming and their individual stage of development within the container.

9:2 Preparation of Grain Spawns.

100g quantities of grain (cereal grains, rye, wheat, milo or millet) in 500 ml Erlenmeyer narrow-neck flasks, were initially washed in a stream of cold water to remove dust, chaff and split grains, and then drained. To the individual flasks was added 30 ml water and 1.65g $CaSO4.2H_2O$ plus 0.42g $CaCO_3$. The grains were heated to boiling on a hot-plate and allowed to stand at room temperature for 24-48 hrs., followed by autoclaving at 15 p.s.i. for 1½ hrs in order to thoroughly cook the grain.

It is necessary to frequently shake the grain during heating, before, and after cooling to thoroughly disperse the chalk and gypsum and thereby prevent sticking of the grains.

Boiling followed by incubation, prior to autoclaving, eliminates a number of contaminants with resistant spores. The flasks were plugged with non-absorbent cotton-wool and capped with aluminium foil following boiling, which prevented excessive wetting of the bungs during extended autoclaving and reduced water-loss from the grain during cooling. The volume of water added to the wet grain was calculated to give a final moisture level in the grain of about 40-50 per cent. The quantities of chalk and gypsum used give a final pH of about 6.5 (Stoller 1962).

The grain was inoculated with 5-8, 1.8 cm mycelial-agar discs from cultures grown on 2% malt agar. The flasks were further shaken about a week following inoculation to disperse newly colonised grain, and again following incubation (4-6 wks. at room temperature) in order to break-up the spawn and thereby prevent excessive intergrowth and tissueing within the grain mass.

10:0 EXPERIMENTS WITH THE GAS DIFFUSION COLUMN.

The diffusion column technique of Griffin, Nair, Baxter and Smiles (1967) was used. The column differed somewhat from the type detailed by them, was constructed in brass 72" x 5" diameter and fitted with 17 horizontal parts in duplicate along its length which allowed sampling of specific gas concentrations throughout the range employed. The individual ports were designed with two pairs of vertically opposed outlets and a single terminal horizontal outlet. In later experiments the top outlets were made serviceable by extending them with the use of thick plastic tubing and retaining the specimen tubes on two racks disposed along the length of the column. Silicone rubber 'suba-seals' were inserted into one set of the horizontal outlets to enable direct sampling from the individual ports. This arrangement allowed for a maximum of 9 culture vessels per gas concentration.

Specimen tubes (1" x 6") were used as culture vessels each containing 20 ml glucose-asparagine agar or 2% malt agar, laid flat to form a thin layer along the length of the tube. 2% agar addition was used in order to give the agar sufficient strength. The tubes were inoculated centrally with 6mm inocula and colony extension measured along the agar surface at regular intervals. Replicates were in two or three for each species at the individual gas concentrations, as indicated.

Gas was supplied from commercial cylinders of nitrogen (containing c. 0.3% oxygen), oxygen and carbon dioxide, plus the use of atmospheric air. Where carbon dioxide was required to be removed from the air, it was passed through 10% NaOH and washed through distilled water. Standard gap meters were used to regulate the volumes of gas passing to the displacement vessels and where necessary calibrated by displacement of water.

The column was allowed to stabilise between 8-12 hrs prior to use. At steady-state, the gas concentration at any position x, along the cylinder, is determined by the formula

$$Cx = Cm - \frac{x}{l} (Cm - Cn)$$

where l is the length of the cylinder and Cm and Cn are the individual gas

concentrations in the displacement chambers at either end of the cylinder.

A calibration curve was obtained over the range of carbon dioxide concentrations 0-1.0% by use of a Fisons Mini gas chromatograph. The values obtained agreed well with theoretically derived values (Figure 15.6).

Individual experiments were designed to measure the growth response in the oxygen range 0.3-21.0% and carbon dioxide ranges 0-5.0% and 0-12.0%

PLATE 8.

GAS - DIFFUSION COLUMN.

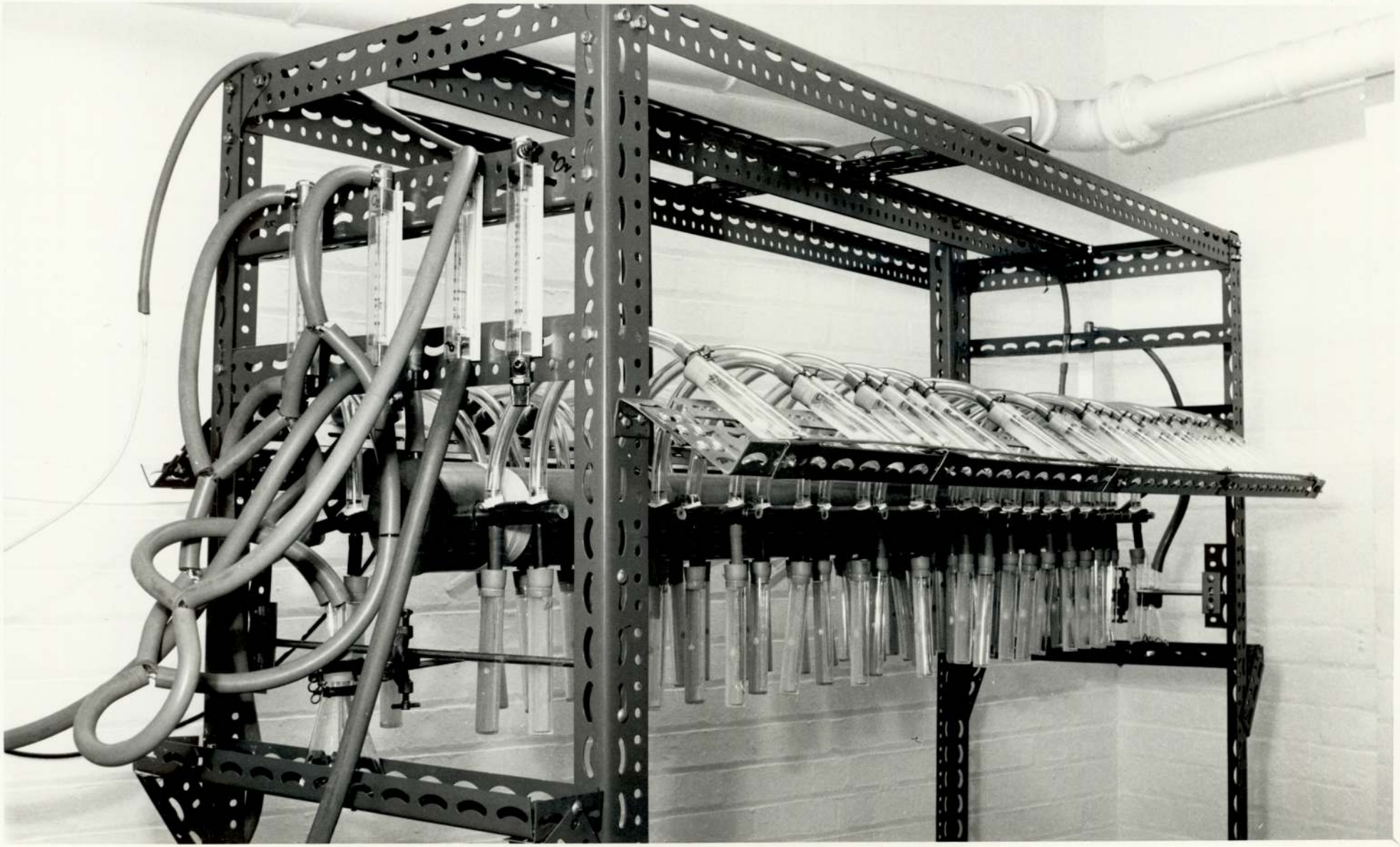
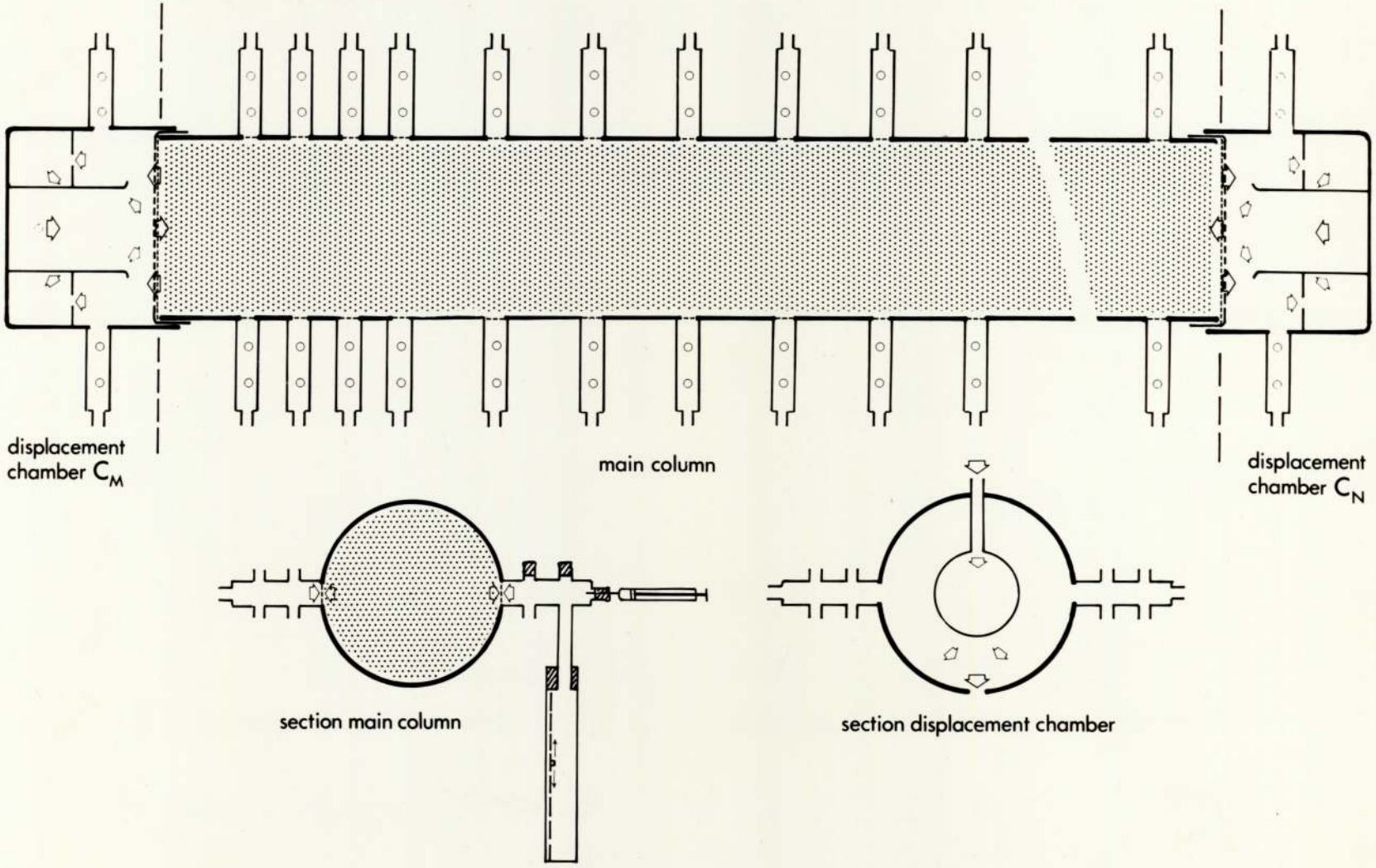


PLATE 9.

DIAGRAMMATIC SECTION

THROUGH GAS-DIFFUSION

COLUMN.


displacement chamber C_M
main column
displacement chamber C_N
section main column
section displacement chamber

11:0 STATISTICAL ANALYSIS OF RESULTS

11:1 Derivation of Least Significant Difference from Analysis of Variance and Standard Error of the Mean.

Least Significant Difference (L.S.D.) was determined by:

L.S.D. = $\sqrt{(2)}$ x t x S.E.M. where Standard Error of the Mean.

$$(S.E.M.) = \sqrt{\frac{\text{error mean square}}{\text{number of samples for each mean.}}}$$

t = Students t at 5.0, 1.0 and 0.1 per cent. Levels of significantce with $n - 1$ Degrees of freedom (^{o}F)

11:2 Standard Error of the Mean (S.E.M.) Determinations.

Obtained from the formula:

$$S.E.M. = (\varepsilon) = \sqrt{\frac{\sum (m-M)^2}{N(N-1)}}$$

11:3 Analysis of Variance Determinations.

(Bishop 1966)

Calculations of Error Mean Square were determined from:

(1) Analysis with three or more replicated treatments.
(2) Analysis with two experimental factors and replication.

R E S U L T S

SECTION 1 RESULTS

1:0 COLONY CHARACTERISTICS

Lepista nuda

Lepista nuda possessed hyphal cells of long, thin, dimensions with a diameter of c. 3 μ (Modess 1941); cells possessed a regular distribution of clamp-connections at each septum.

Isolates of L. nuda were readily produced from pileus and stipe tissue blocks in a fresh condition. The hyphae grew readily from the upper portion of the block to give a largely aerial, loosely woven colony with a distinct violaceous tint. A strong purple colour was produced by aggregation and compression of the aerial hyphae such as occurred following manipulation with a needle or removal of a sample with a cork-borer. Following three to four mycelial transfers the colonies became considerably less aerial. Cultures obtained from the Central Bureau voor Schimmelcultures, Netherlands, isolated in 1948 and 1958 were producing adpressed and submerged colonies on agar media.

Regularly subcultured colonies of L. nuda produced stable and consistent growth, generally of a fine and dispersed nature though this character varied to some extent over the range of isolates. Sectors or secunds were not produced during normal culture conditions, although light tufts of aerial hyphae and in some instances compact areas of deeply lilaceous hyphae did arise where nutritional exhaustion or drying-down of the agar medium was taking place. In general none of the isolates readily produced strands on agar media even following extended periods of incubation. The instances where stranding did take place were, (a), following extended incubation on cellulose agar; (b), following growth in flasks on cellobiose media; (c), following growth in flasks, containers, tubes and divided Petri-plates on compost or peat-chalk media, (d), following growth from individual grains of rye grain spawn onto 2% malt agar (for a single isolate No. 8).

Voltz (1966) reported rhizomorph development by his strain of L. nuda on chlamydospore agar, however, this finding could not be repeated with 3 strains of L. nuda on this medium.

Lilaceous colour development in cultures of L. nuda was

largely in response to light although some isolates (5, 7, 8 and 10) produced stronger colouration or were prone to sporadic production of dark colouration, those cultures maintained in a dark incubator or in a coldstore at 4°C in darkness were generally white or sporadically coloured compared to the more even development of colour with colonies maintained under conditions of natural or artificial lighting. This was also the case with respect to the L. saeva isolates. Strong spawn growth of isolates in flasks and containers through the surface peat/chalk casing layer resulted in strongly coloured purple, tissued mycelium at the casing surface.

Growth of grain-spawns was generally good. Once colonised the mycelium became increasingly denser, this could lead almost to the development of tissueing, where cultures were maintained in the coldstore over long periods of time (this was particularly strong for one strain, No. 13).

Good growth was produced by all strains on 2% malt extract agar(Boots Pure Drug Co.) (MEA), more luxuriant growth resulted when this was supplemented with either casein hydrolysate (CMEA), mycological peptone (PMEA) or yeast extract (YMEA) at 1g/litre. Growth of these isolates on potato dextrose agar (Oxoid Ltd.) and chlamydospore agar (formula of Voltz 1966) was very poor, resulting in brown discolouration restricted aerial development and on chlamydospore agar submerged development (probable effects of the very low level of nitrogen in these media).

Growth of isolate 1 on Treschow Wort agar (Treschow 1944), Lutz (Singer 1962) medium and Hagem Malt agar (modified by Modess 1941) was generally inferior to malt agar. Growth of a variety of L. nuda isolates on the latter was very restricted, poor growth was probably due to unavailability or direct inhibition by the iron source.

Lepista saeva

Hyphae of L. saeva were wider and more branched than those of L. nuda, clamp connections were clearly observable on individual septa. Hyphae grew readily from tissue blocks, however, isolates of this species were considerably less aerial and more compact,

P L A T E 10.

Strong strand development by Lepista saeva.
Isolate 6B following growth on malt/yeast extract agar in flasks.

SCALE 1 cm.

forming denser and slower growing colonies, though of a very uniform overall appearance.

Development of purple-lilaceous colouration was particularly strong only in some colonies, in these, however, the colonies were darker, possibly due to denser development of the surface hyphae. An isolate maintained in culture for a long period (C.B.S. 355.33) developed adpressed surface growth.

Stranding generally developed on substrates similar to those described for L. nuda. L. saeva isolate 6B grown in flasks on YMEA produced very strong development of strands, this was particularly so where in several of the flasks, perpheral points of inoculation (produced accidentally) developed strongly and linked with the centrally developed colony arising from the inoculation plug.

Growth of grain spawns following colonisation became very dense, in particular where these were maintained at 4°C in the coldstore, the density of development produced purple tissued regions, tissueing took place strongly at the surface of the grain spawn. Such spawns could not be readily broken-up and removed from the flask. Colonies produced by isolates 248.69, 5 and 6B in stationary liquid culture were particularly dense.

L. saeva cultures were generally more susceptible to staling with increasing colony age and at temperatures higher than optimal.

Colonies produced by isolates 5 and 6B on potato dextrose agar were brown and restricted, chlamydospore agar produced very light surface to sub-surface development. Growth of L. saeva isolate 1 on Treschow Wort agar, Lutz and Hagem agars was generally inferior to 2 % malt agar. Malt agar supplemented with 0.1% Casein hydrolysate, peptone or yeast extract afforded heaver colony development.

Calocybe gambosa

Hyphae of C. gambosa were heterogeneous in size though wider than those of the Lepista spp.,. Hyphae under magnification

frequently appeared aggregated into strand-like formations.

On a few natural media isolates 1 and 2 particularly the former developed a 'bushy' appearance where the aerial hyphae appeared to be composed of short 'asbestos-fibre' like strands, these out-growths in particular arose from the inoculum plug where autolysis or staling of the media had taken place. Growth of C. gambosa isolate 1 as wheat-grain spawn produced these spiked 'bushy' out-growths. Newly isolated mycelia particularly strains 3, 4 and 5 produced submerged hyphal developement. Isolates 1 and 2 were routinely cultured, newly isolated hyphae grew very slowly, a large number of mycelial transfers were required to enable adaptation to the medium and ensure, uniform and repeatable colony growth. For this reason the later isolated strains 3, 4 and 5 were not used regularly. Irregular culturing resulted in long lag periods before growth was resumed.

C. gambosa was prone to the occassional formation of rapidly growing sectors. This occurred with a number of flasks receiving acetic, butyric or valeric acid supplements to the standard glucose-asparagine medium in stationary liquid culture and on occasional Petri-plates where the additions were the fatty-acids, oleic, stearic and palmitic acid.

All strains produced a yellow pigment on 2% malt agar and particularly strongly on chlamydospore agar. The culture fluid from growth on the glucose-asparagine medium was generally bright yellow-orange. The production of pigment varied with the carbon or nitrogen source used in stationary culture work. Freshly isolated strains noteably 3, 4 and 5 produced large numbers of orange stellate crystals over the surface of the agar medium particularly on rich media. Isolates 1 and 2 produced very light development on chlamydospore agar but good development on potato dextose and malt extract agars. Favourable growth with these media indicates good utilisation of starch, but also possibly that a high C/N ratio is favourable to growth. Strong development with these media may also reflect the presence of growth stimulating substances (e.g. amino-acids or peptides) within the media. Growth on unamended glucose-asparagine medium was generally poor.

TABLE 1:1 Comparison of Growth of single Isolates on a range of Solidified Media.

Natural/Semi-synthetic Media	LEPISTA NUDA 1 mm/day	LEPISTA SAEVA 1 mm/day
HAGEM MALT AGAR (Modess 1941)	0.95	1.0
TRESCHOW WORT AGAR (Treschow 1944)	0.81	2.46
LUTZ MALT AGAR (Singer 1962)	1.63	2.23
MALT EXTRACT AGAR (MEA)	2.28	3.53
MALT EXTRACT + CASEIN HYDROLYSATE AGAR (CMEA)	2.54	4.33
MALT EXTRACT + YEAST EXTRACT AGAR (YMEA)	2.3	4.21
MALT EXTRACT + PEPTONE AGAR (PMEA)	2.59	4.45
NUTRIENT AGAR (NA)	1.8	1.08

Yeast Extract, Casein Hydrolysate, Peptone supplements at 1g/L to 2% Malt extract Agar.

Figures average of 5 replicates for 2 colony diameters at right angles per colony.

1:1 Combined Studies in Divided Petri-Plates and in Mixed Cultures

Inhibition of fungal colony extension by sirgle bacterial isolates from each soil was due to a certain proportion of bacteria within the spectrum isolated. The effect was most evident with isolates from the lighter sandy soils (Coleshill, Droitwich and Stagborough Hill) particularly in C. gambosa strains than for isolates obtained from the heavy clay soils (Moreton Bagot and Evington Meadows).

Rapid, light mycelial development occurred over the soil extract medium, in general, addition of cell-free solutions of the five natural soils strongly inhibited growth off the inoculaton plug particularly the 4 and 6 ml additions. The L. saeva isolate was tolerant of the lower level of addition of the cell free solution. The solution obtained from the'casing-soil' flora was considerably less toxic in its effect, it produced no growth inhibition of the L. saeva isolate.

Treatments (a), (b), (c), (d), (e) and the use of mixed bacterial inoculation plugs, had no direct morphological effect on growth (barring some inhibition of growth in the vicinity of the bacterial inoculation plug). Colonisation was light, though somewhat stronger and more uniform with treatments (c) and (d). Only L. nuda isolate 13 strongly colonised all treatment halves. Light strand development occurred with L. nuda isolates on (b) and (d), isolate 13 formed a strand network on medium (e). L. saeva isolate 6B formed light stranding on medium (d). Development of strands generally originated and was strongest in the aerial hyphae passing over the intermediate dividing wall of the Petri-plate. Stranding was also often obvious radiating from the inoculation plug on the malt agar section of the plate.

SECTION 2

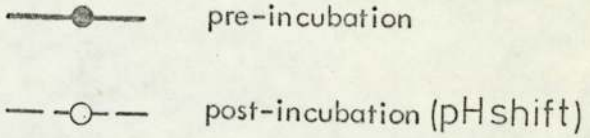
2:0 EFFECT OF HYDROGEN ION CONCENTRATION ON GROWTH

L. nuda isolates showed a pH optimum at approximately 7.0 with a shift over the 21 day incubation period to more acid conditions in

EFFECT OF HYDROGEN ION CONCENTRATION ON GROWTH

3 WEEKS INCUBATION AT 25°C

FIGURE 2.1



Lepista nuda **7**

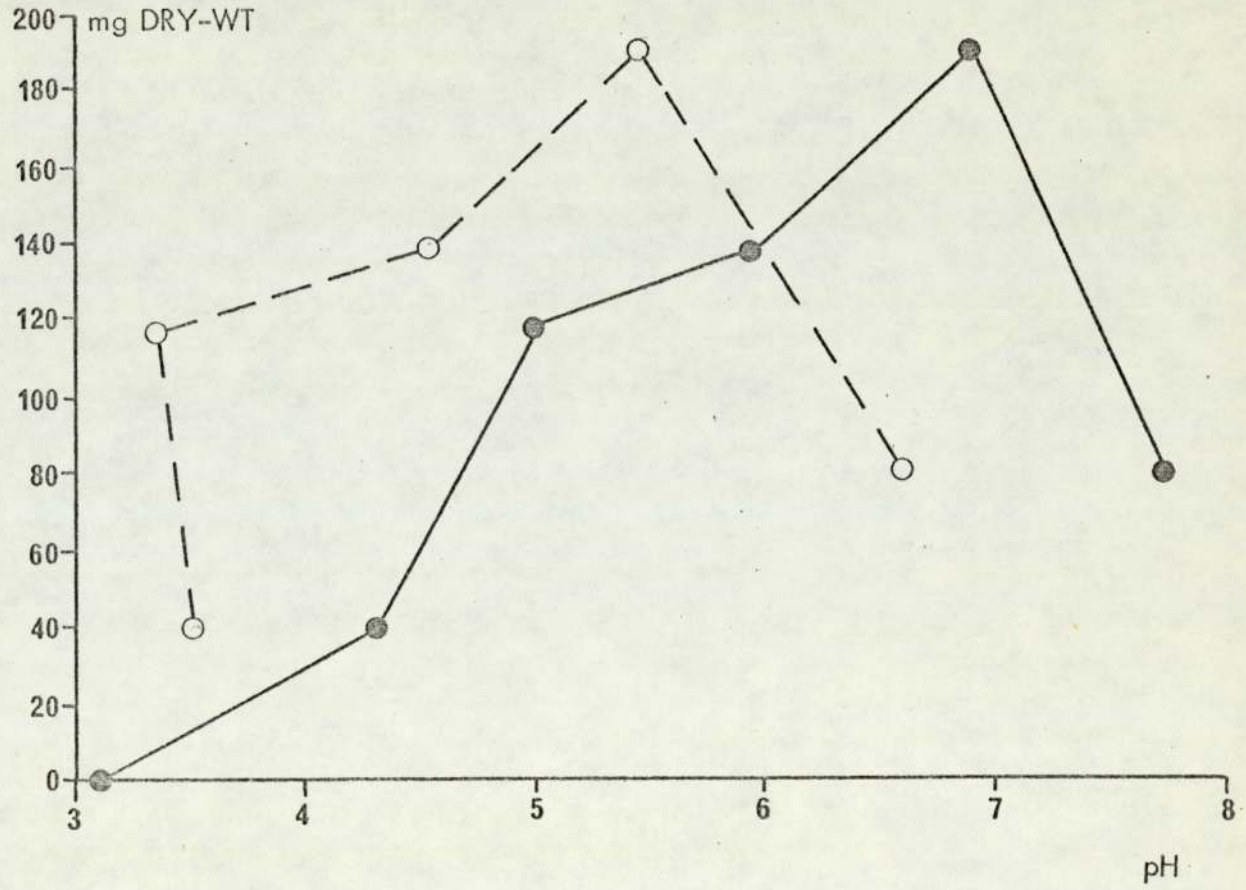


FIGURE 2.2

EFFECT OF HYDROGEN ION CONCENTRATION ON GROWTH

Lepista nuda 6

3 WEEKS INCUBATION AT 25°C

pH CULTURE FLUID
pre-incubation
post-incubation
(pH shift)

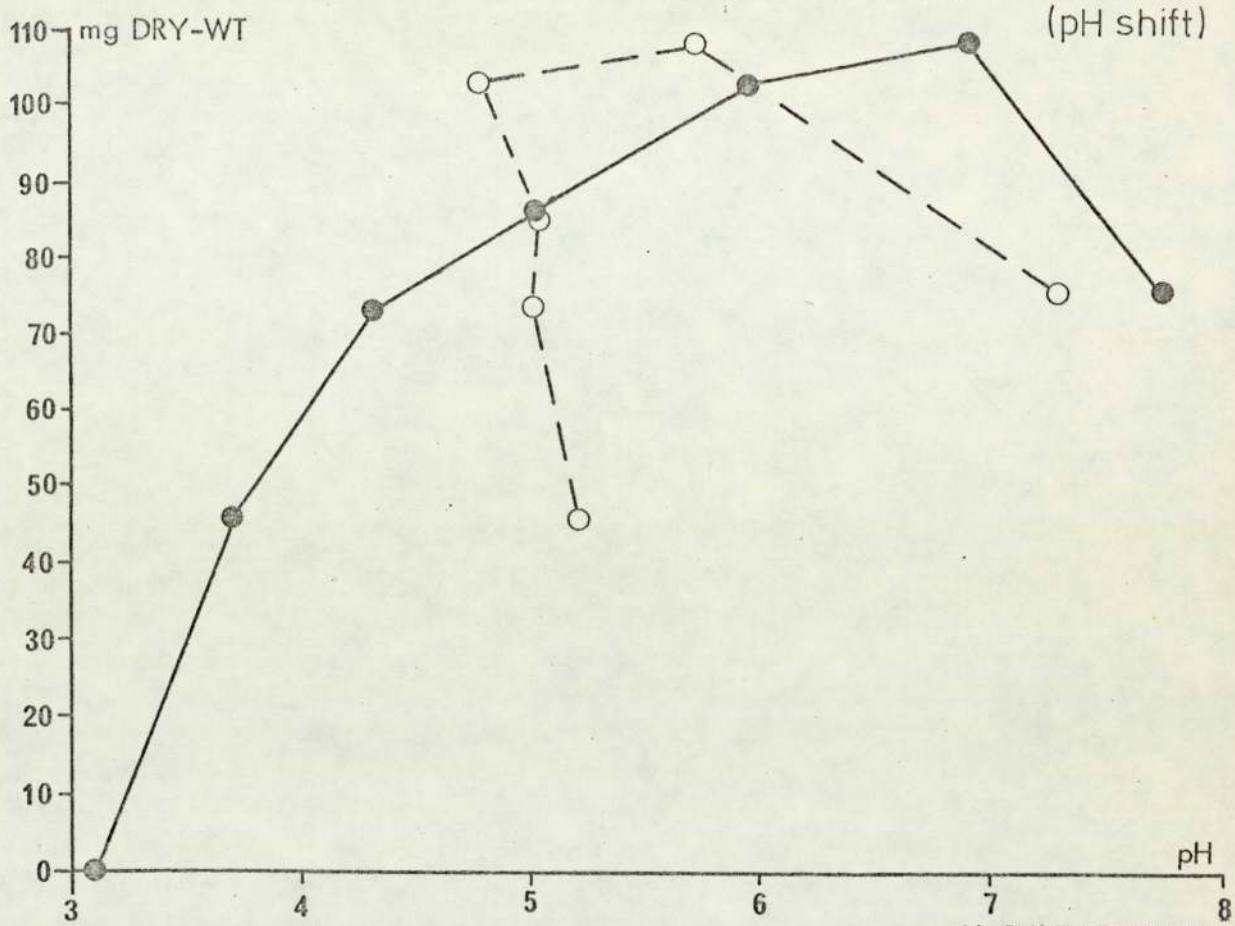
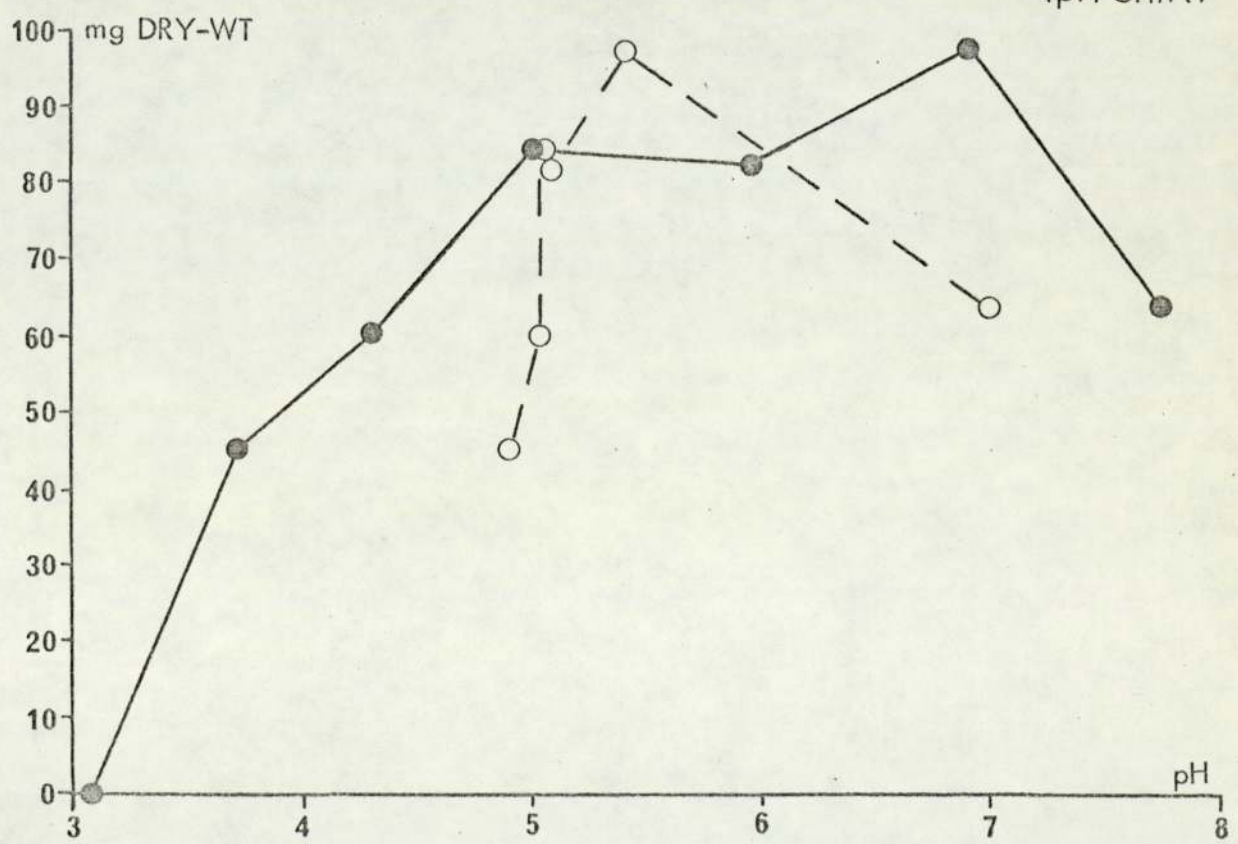


FIGURE 2.3

Lepista nuda 8

3 WEEKS INCUBATION AT 25°C

pH CULTURE FLUID
pre-incubation
post-incubation
(pH shift)



EFFECT OF HYDROGEN ION CONCENTRATION ON GROWTH

3 WEEKS INCUBATION AT 25°C

FIGURE 2.4

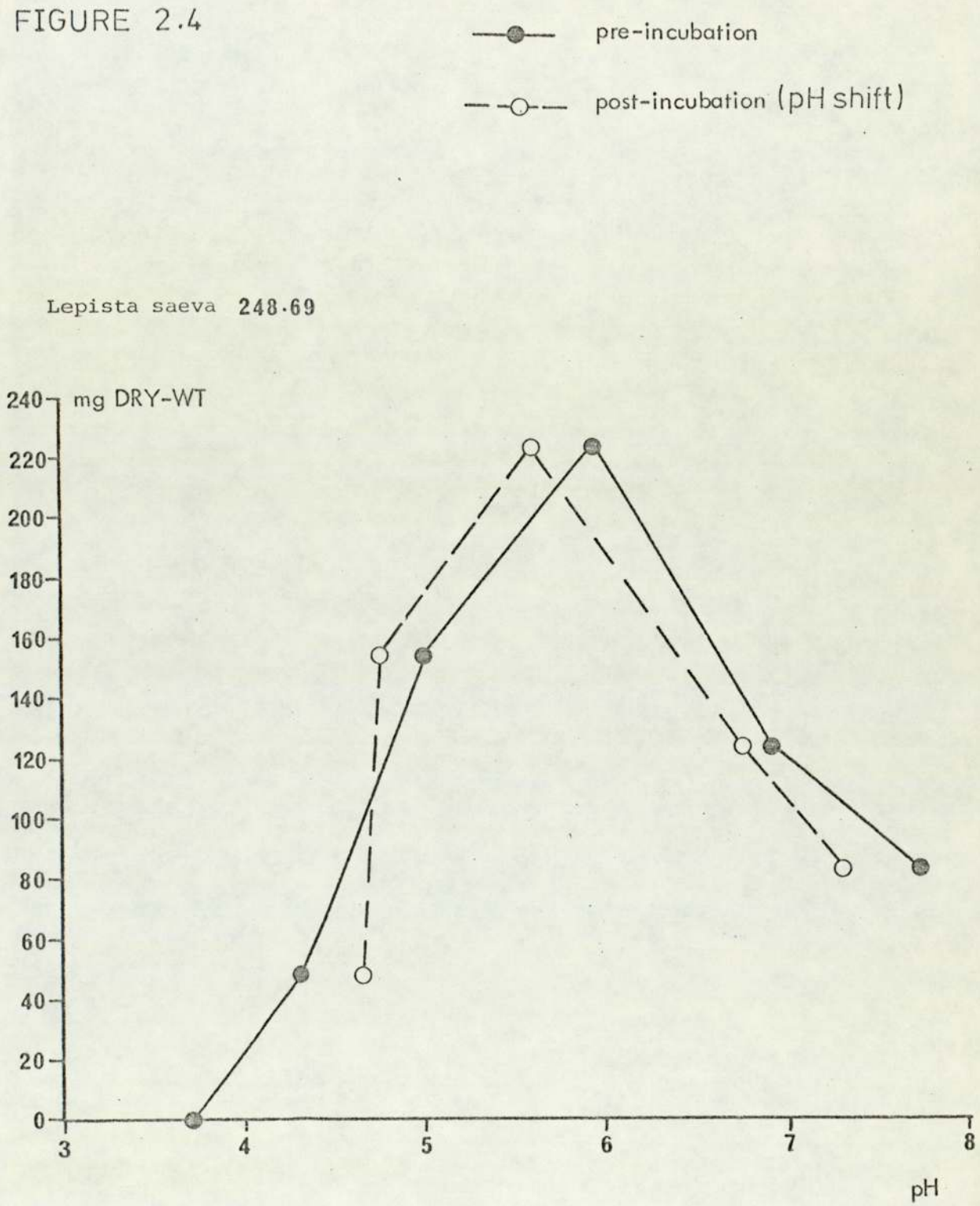


FIGURE 2.5

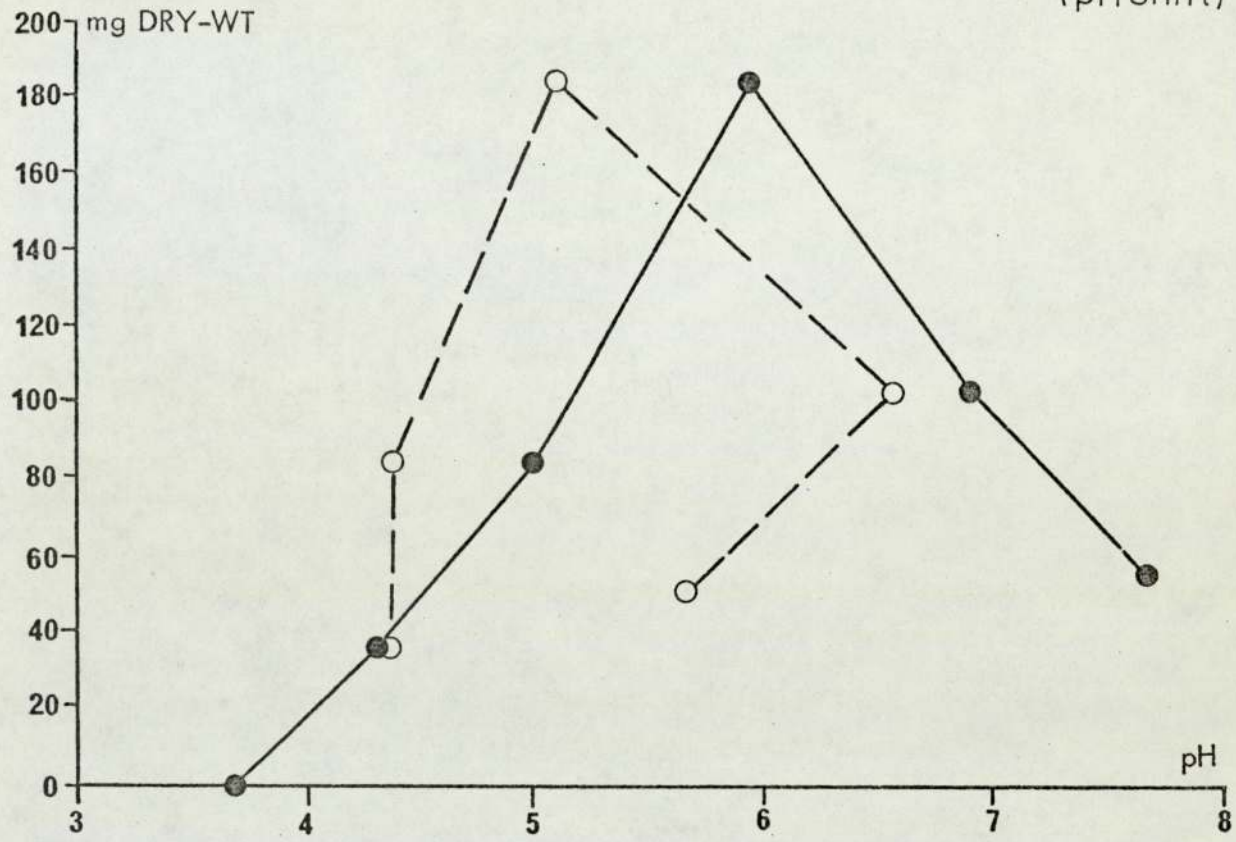
EFFECT OF HYDROGEN ION CONCENTRATION ON GROWTH pH CULTURE FLUID

3 WEEKS INCUBATION AT 25°C —●— pre-incubation

—○—post-incubation

(pH shift)

Lepista saeva 5



pH CULTURE FLUID

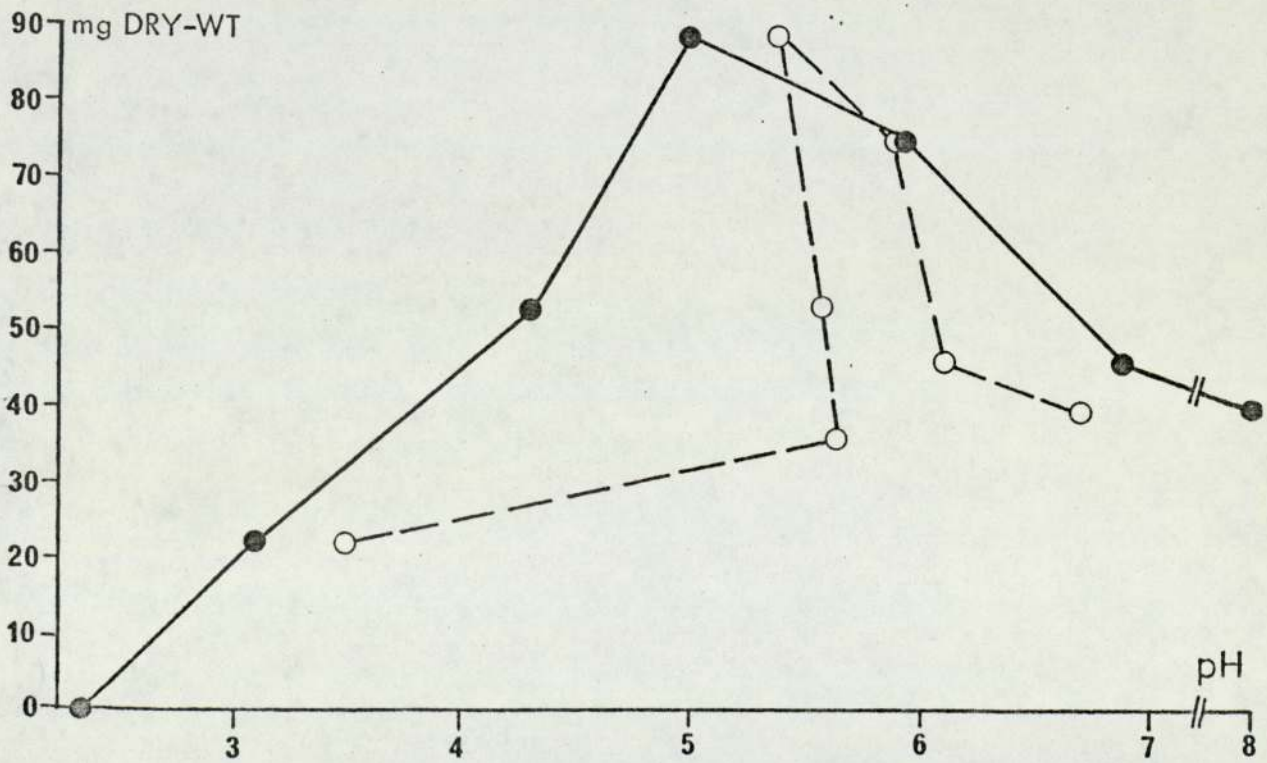
3 WEEKS INCUBATION AT 25°C —●— pre-incubation

—○—post-incubation

(pH shift)

FIGURE 2.6

Lepista saeva 6A



EFFECT OF HYDROGEN ION CONCENTRATION ON GROWTH

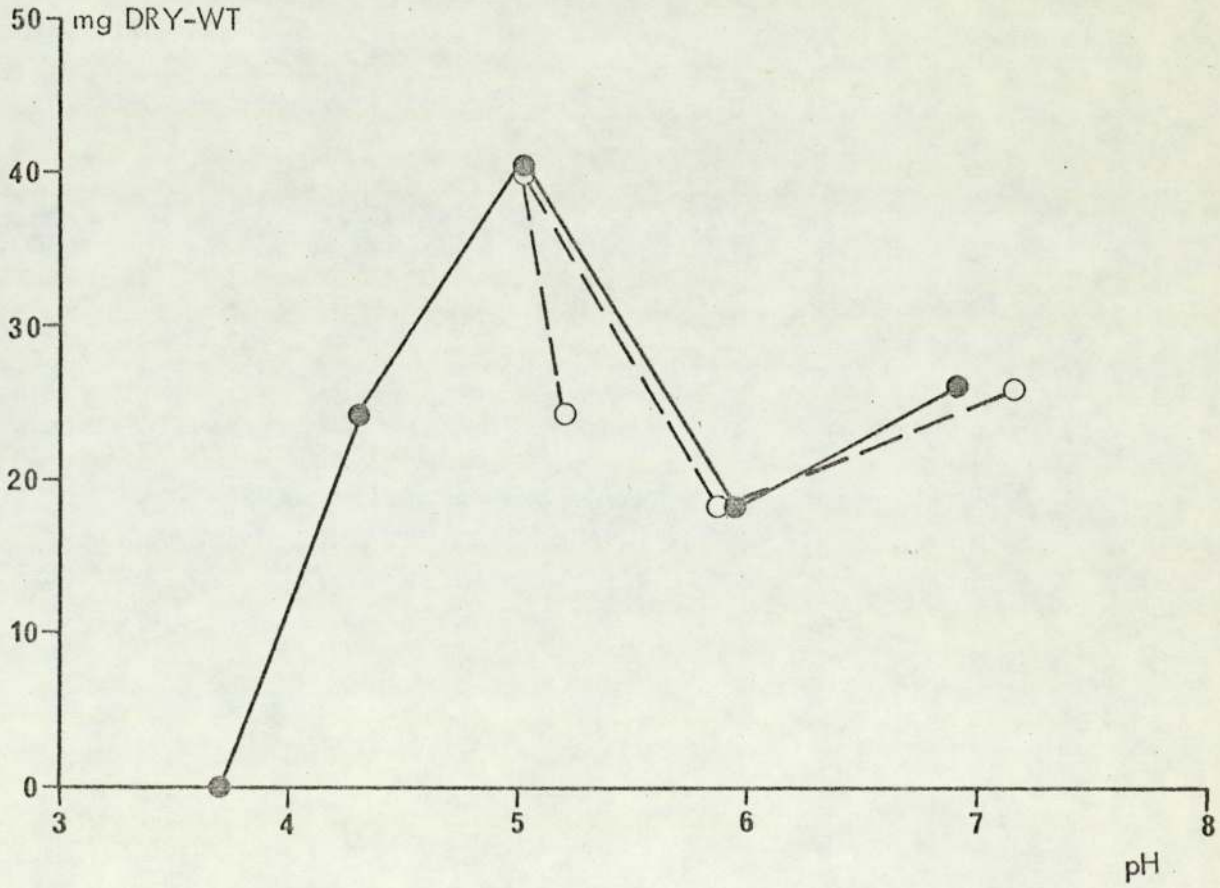
3 WEEKS INCUBATION AT 25°C

FIGURE 2.7

pre-incubation

post-incubation (pH shift)

Calocybe gambosa 1



the region of pH values 5.0 to 6.0 (Figures 2.1, 2.2 and 2.3). Isolates 6 and 8 were more tolerant of acid conditions than isolate 7, growth, however, was only prevented for all isolates at pH 3.1. Growth was not strongly inhibited in the alkaline range due probably to a rapid shift in pH to more favourable acid conditions. In general there was a shift in pH values to terminal values in the range 5.0 to 7.0.

L. saeva isolates indicated a more distinct optimal value within a narrow zone of pH preference between 5.0 to 6.0 (Figures 2.4, 2.5 and 2.6). The alteration in pH values following incubation was not as marked as L. nuda. Isolates were less able to tolerate acid conditions, growth for 2 isolates (248.69 and 5) was inhibited at pH 3.7, however, isolate 6A was able to tolerate values down to 2.35.

Growth of a single isolate of C. gambosa (2) (Figure 2.7) was optimal at pH 5.0. Growth was inhibited at pH 3.7. The pH shift was generally to more alkaline conditions.

The growth of all three species was good in these millipore filtered solutions where the formation of inhibitory substances or breakdown products frequently a product of media autoclaving was prevented. Excellent growth of the C. gambosa was made in millipore filter sterilised solutions.

3.0 EFFECT OF TEMPERATURE ON GROWTH

Of the four isolates of L. nuda used in this study two demonstrated an optimum at 25°C, two at 20°C (Figure 3.1). Those isolates with the lower temperature optima grew better at 4, 10 and 15°C. All isolates were inhibited by a temperature of 30°C.

Growth of the three isolates of L. saeva tested was optimal at 20°C (Figure 3.2). Good growth was generally attained at temperatures of 10 and 15°C. Slight growth of all isolates occurred at 4°C. Growth of all isolates was inhibited at 30°C.

Temperatures of 27 to 28°C had a marked effect on growth

FIGURE 3.1

EFFECT OF TEMPERATURE ON GROWTH
21 DAYS INCUBATION

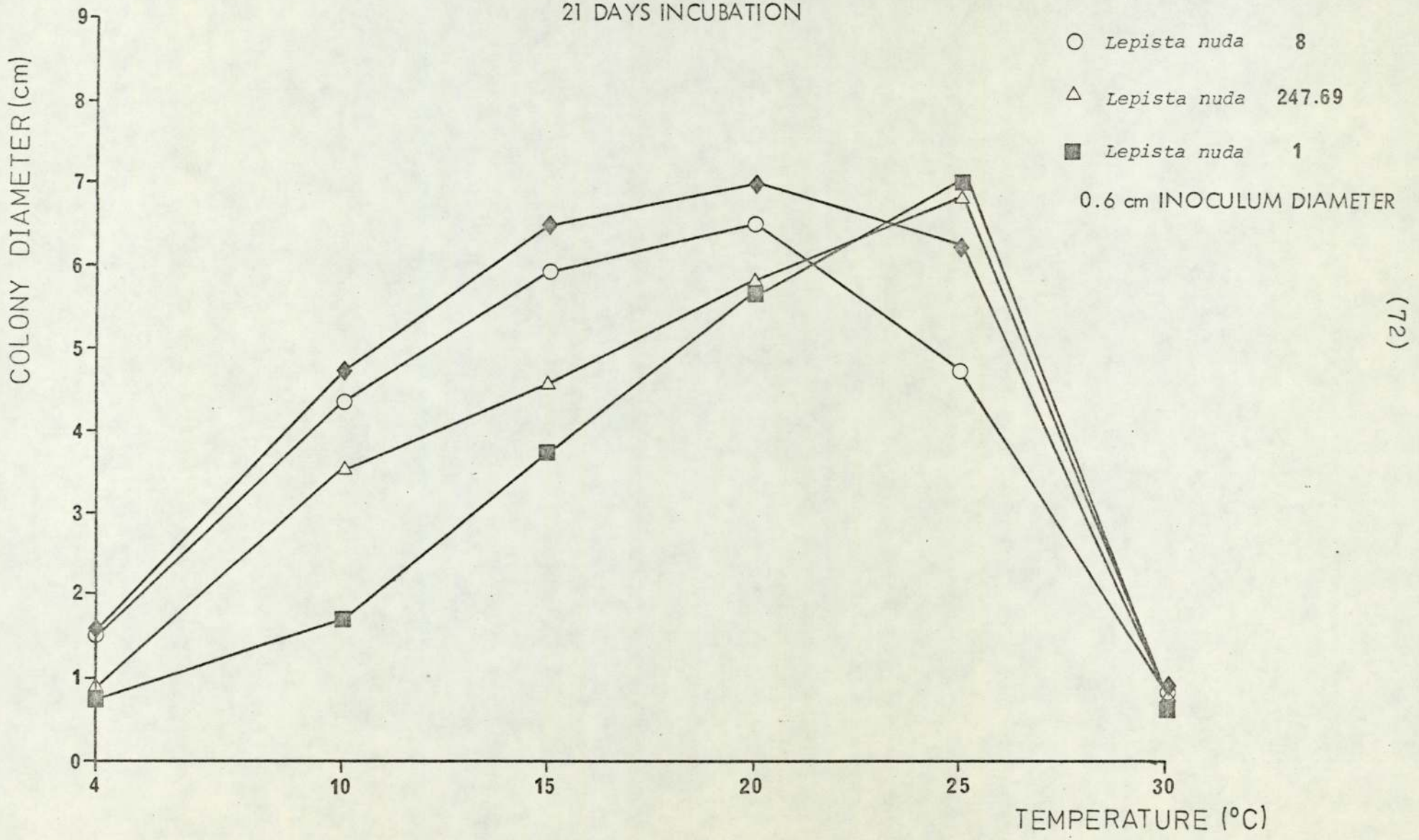


FIGURE 3.2

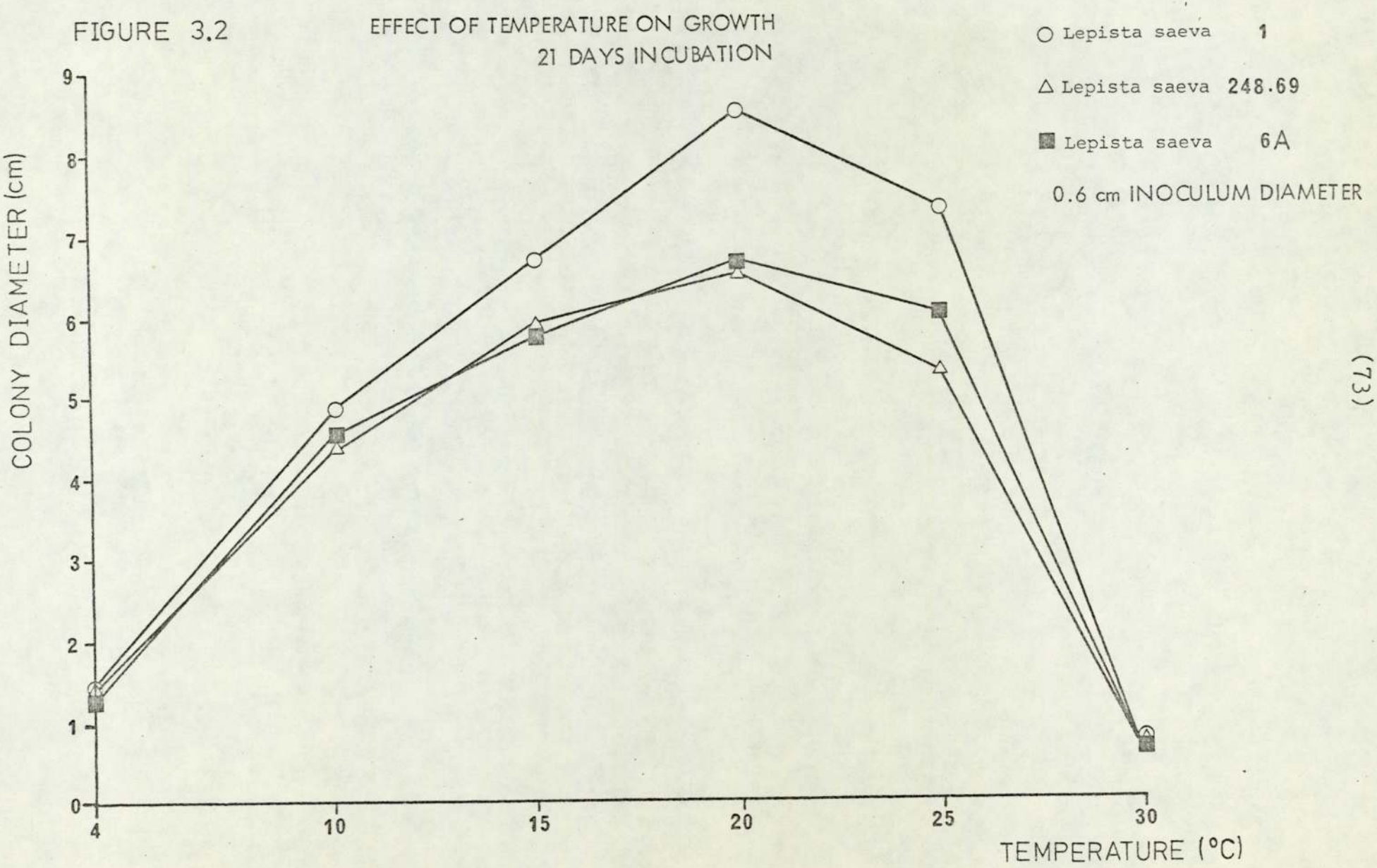
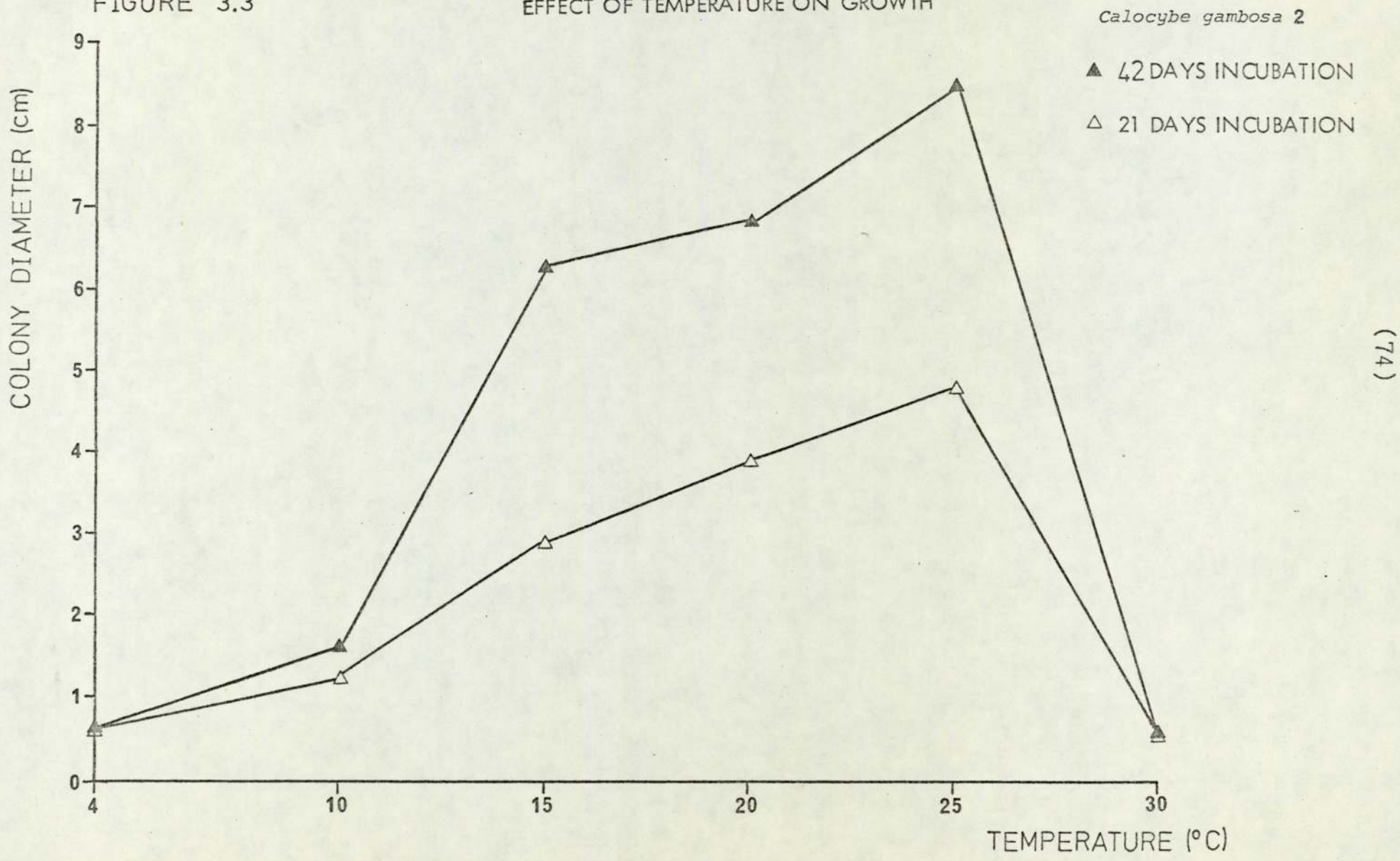

EFFECT OF TEMPERATURE ON GROWTH
21 DAYS INCUBATION
○ Lepista saeva 1
△ Lepista saeva 248.69
■ Lepista saeva 6A
0.6 cm INOCULUM DIAMETER
COLONY DIAMETER (cm)
0
1
2
3
4
5
6
7
8
9
4
10
15
20
25
30
TEMPERATURE (°C)

FIGURE 3.3

EFFECT OF TEMPERATURE ON GROWTH



of L. saeva isolates 1, 5, 6B and 7 and also to a lesser extent with isolate 248.69. Effects were autolysis of the mycelium with the agar medium turning dark brown, growth was difficult to resume from these colonies. Culture staling was not a feature of L. nuda isolates in this temperature range.

Growth of the C. gambosa isolate was optimal at 25°C with good growth at 15 and 20°C (Figure 3.3). Growth was markedly reduced at 10°C and inhibited at 4°C. No growth took place at 30°C.

SECTION 3

4:0 EFFECT OF ASH AND TRACE METALS ON GROWTH

Growth of the species L. nuda and L. saeva was very significantly greater ($P = 0.001$) on the complete medium than that attained on the basal salt solution (with the exception of L. nuda isolate 8) (Figures 4.1 and 4.2).

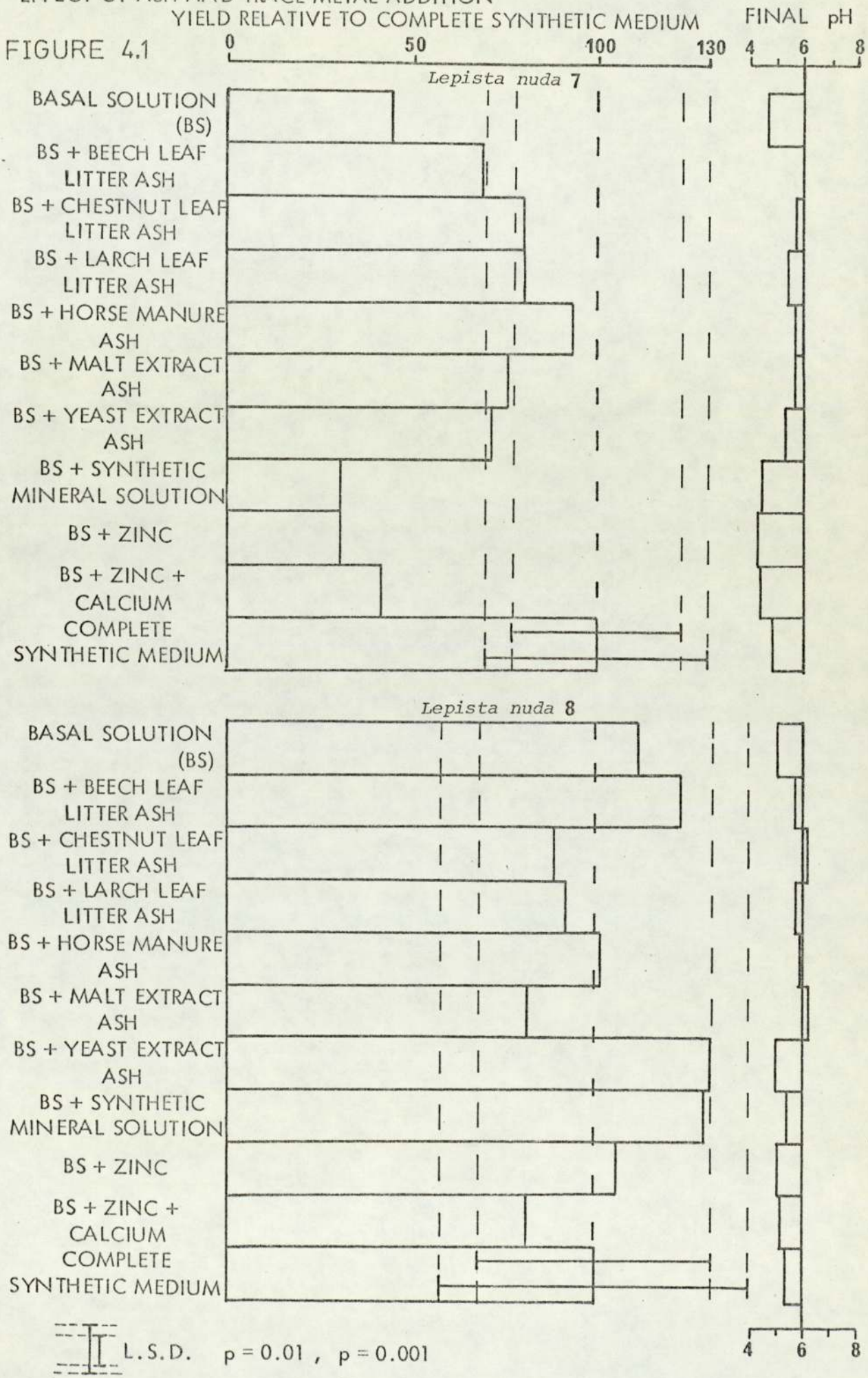
Components of the synthetic mineral solution, Ca, Mo, Cu, Mn and Zn did not significantly improve growth (exception L. saeva 6B), and did not equal the growth promotory effect of yeast and malt extract ash (exception L. nuda 8).

Zinc or zinc and calcium additions did not markedly affect growth, a slight increase in yield with calcium and zinc addition indicates that calcium is important, however, calcium exerts little effect at low concentrations (i.e. as a trace metal). The basal solution with zinc and calcium additions approximated the composition of the complete synthetic solution, with the exception of a greatly reduced concentration of calcium, therefore, the greatly improved yields in the latter are probably due to the ability of calcium to counteract the effects of high levels of metal ions antagonistic to growth or the build-up of inhibitory concentrations of intermediate metabolic products (possibly by chelation) such as certain of the Krebs cycle organic acids.

The beneficial effects of the addition of ash components

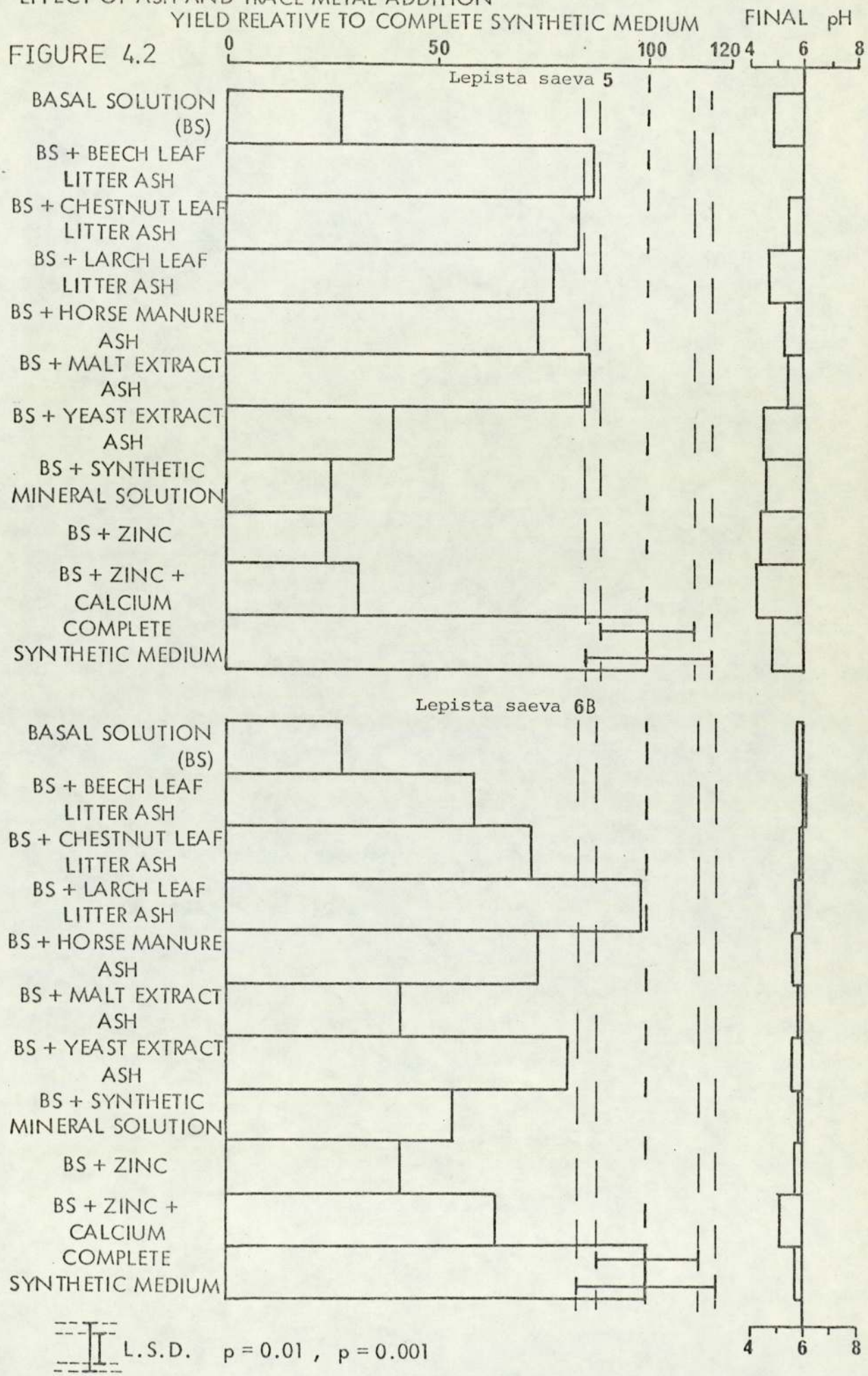
EFFECT OF ASH AND TRACE METAL ADDITION
YIELD RELATIVE TO COMPLETE SYNTHETIC MEDIUM

FIGURE 4.1



EFFECT OF ASH AND TRACE METAL ADDITION
YIELD RELATIVE TO COMPLETE SYNTHETIC MEDIUM

FIGURE 4.2



of leaves and manures was probably due to the calcium component or the addition of other metal ions particularly potassium and magnesium.

The exception-*L. nuda* isolate 8 — yielded higher on the basal solution than the complete medium. It is possible that metal ion components of the yeast extract and synthetic mineral solution were stimulatory to growth of this isolate.

5.0 EFFECT OF VITAMINS ON GROWTH

Growth on the complete vitamin solution minus pyridoxine thiamine, biotin or folic acid and with the vitamin-free control was significantly reduced ($P = 0.001$) within the species *L. nuda* and *L. saeva*, with the exception of *L. nuda* isolate 8 on media minus pyridoxine, biotin and folic acid, this isolate demonstrating a sole requirement for thiamine (Figures 5.1 and 5.2).

All isolates of these species demonstrated a clear requirement for thiamine, the requirement for biotin was only partial.

Absence of folic acid from the complete vitamin solution produced a growth reduction in both *L. saeva* isolates and in the *L. nuda* isolate 7, growth of the latter was also reduced when p-aminobenzoic acid (part of the folic acid moity) was deleted from the complete medium.

Growth of the *L. saeva* isolate 6B was signicantly reduced with removal of calcium pantothenate and niacin from the complete vitamin solution.

Low yields were produced by the *Calocybe gambosa* isolates (Figure 5.3). The cause may have been use of a purified asparagine preparation for the media, also possibly the additions lipoic acid, inositol or choline chloride may have been inhibitory to growth. It is possible that these substances caused a general suppression of yield by the *L. nuda* and *L. saeva* isolates.

Although the complete vitamin solution was formulated as a synthetic yeast extract vitamin source, actual addition of yeast extract had a clearly beneficial effect particularly in the case of the

FIGURE 5.1

VITAMIN REQUIREMENT FOR GROWTH

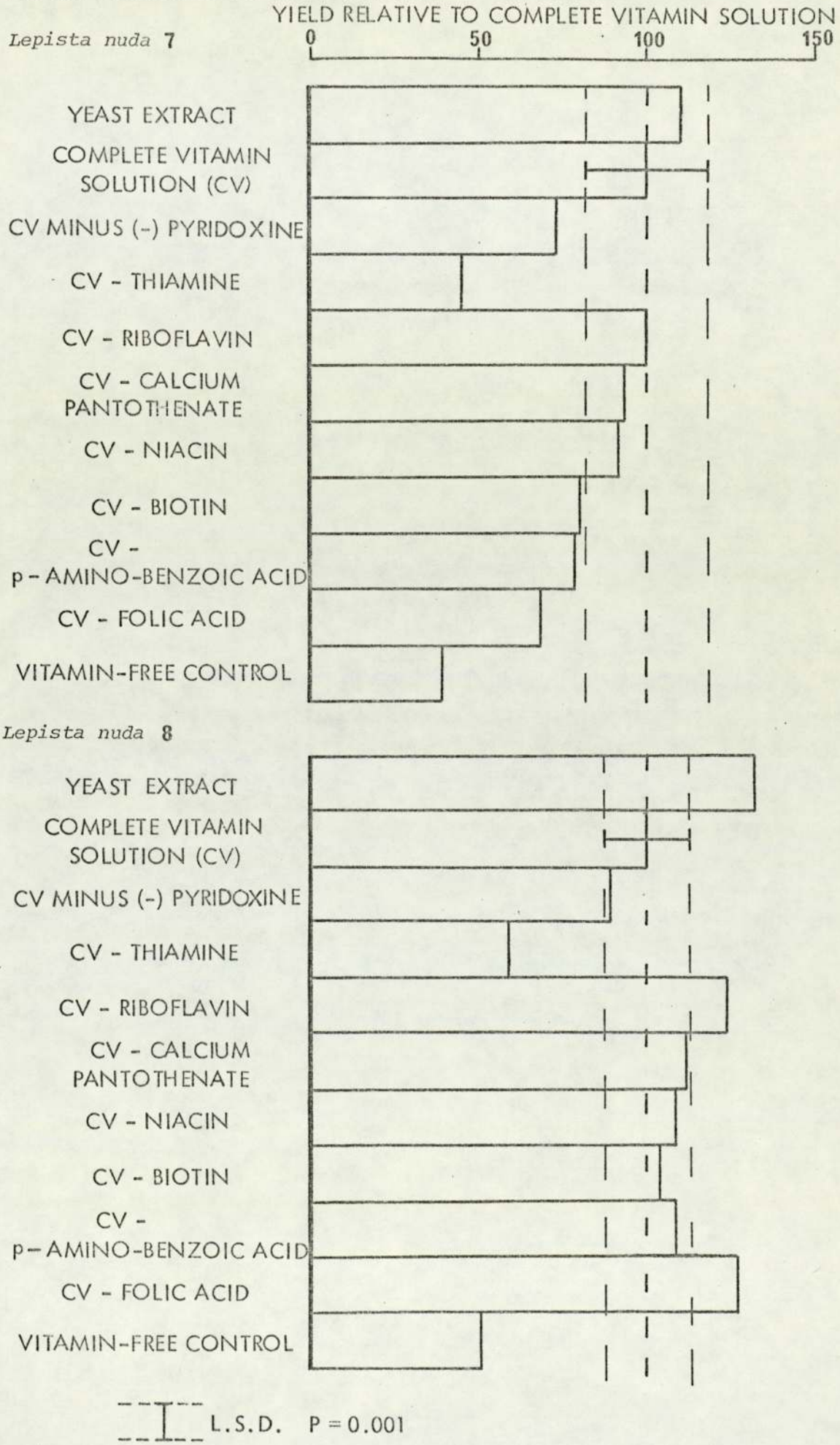


FIGURE 5.2
VITAMIN REQUIREMENT FOR GROWTH

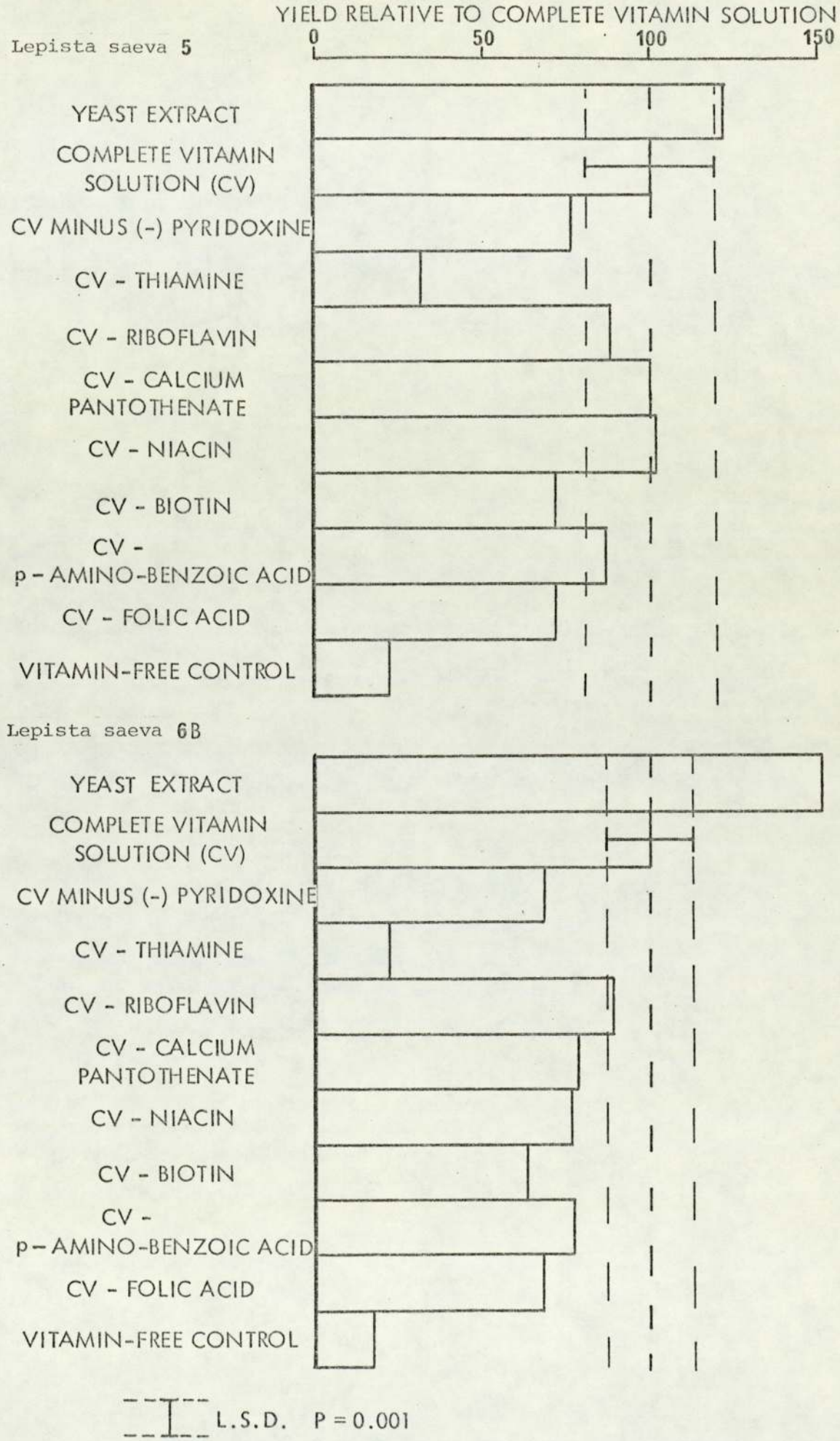
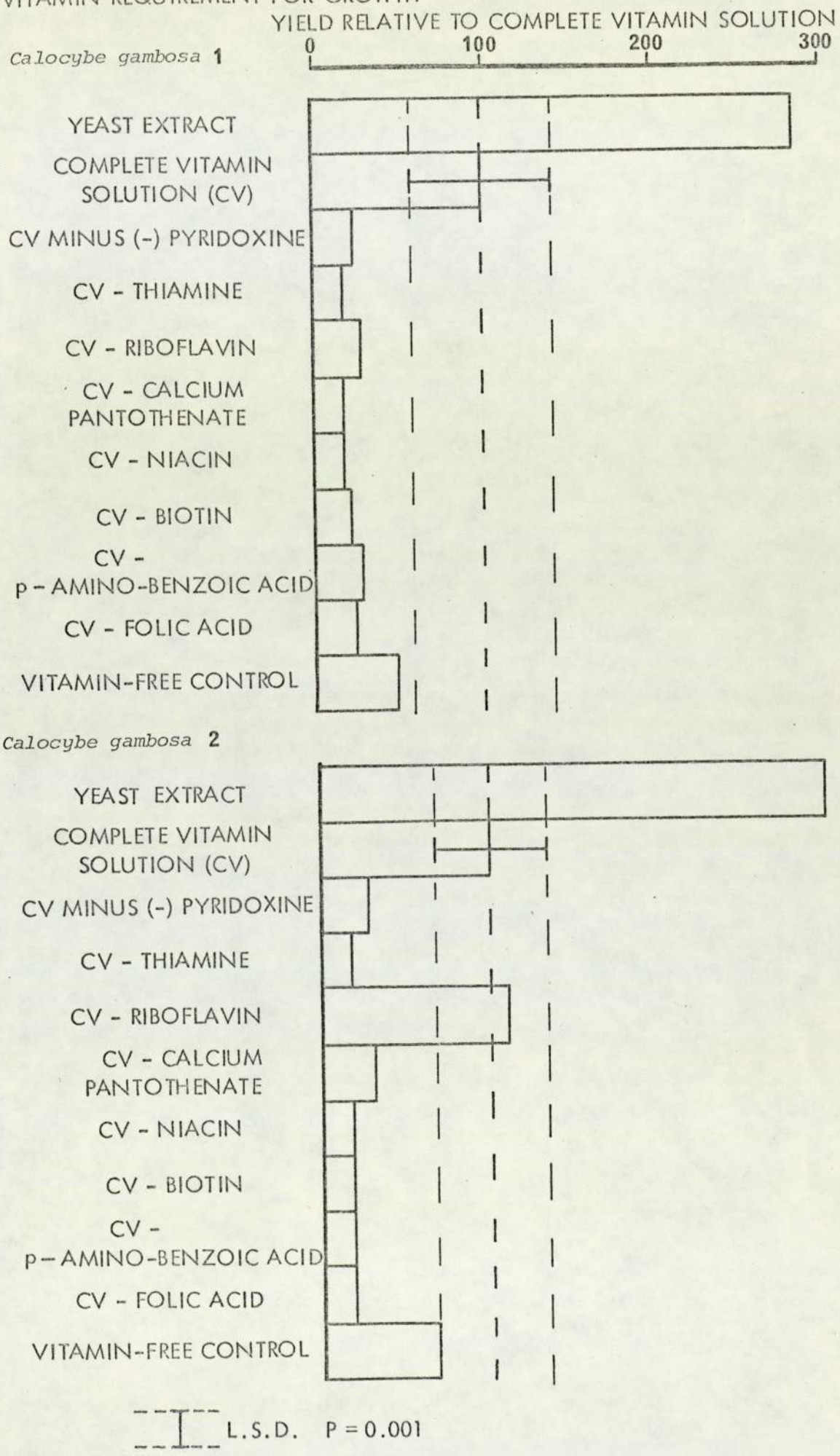


FIGURE 5.3
VITAMIN REQUIREMENT FOR GROWTH



C. gambosa isolates. This may have been due to the addition of amino-acids or other stimulatory substances contained within this source of yeast extract.

6:0 INFLUENCE OF CARBON : NITROGEN RATIO ON HYDROGEN ION CONCENTRATION AND YIELD

In an initial experiment with inadequately buffered media the final yields were influenced by the pH change in the media during growth (Figures 6.1 and 6.2.). At low C/N ratios (9:1) the hydrogen ion concentration shifted into the alkaline range, this was particularly marked in the case of the L. nuda isolate. With increase in sugar levels acids formed markedly reduced pH to low values, this change was strongest with increasing concentration of the carbon and nitrogen sources in the media.

In the second experiment yields were assessed at seven day intervals (together with the pH values of the pooled culture fluid) to 28 days. An increased level of phosphate buffering was employed, pH values did not alter markedly for either of the Lepista isolates, the greatest drop recorded was for the L.saeva isolate following 28 days incubation at 0.3% asparagine (0.5592 g%N) addition. (1.9 units) (Appendix Table 6.5-6)

Considering yields of the L. nuda isolate at 0.1 and 0.3 per cent additions of asparagine, (Figures 6.3 and 6.4) higher dry-weights were produced with increasing the C to N ratio. The best yield at 28 days for the 0.1% asparagine series was at a C/N ratio of 21:1.

A C/N ratio of 21:1 was also the most favourable ratio until 21 days incubation at the higher nitrogen level (0.5592g%N), at this point with nitrogen in excess a shift in the carbon level reduced the ratios of 25 and 28:1 to a more favourable balance enabling increased yields.

Yields of L. saeva for the 0.1864 g%N series (Figure 6.5) showed carbon limitation (assumption that mycelium contains

FIGURE 6.1

EFFECT OF CARBON : NITROGEN RATIO ON YIELD

GROWTH AT FOUR CONCENTRATIONS OF NITROGEN

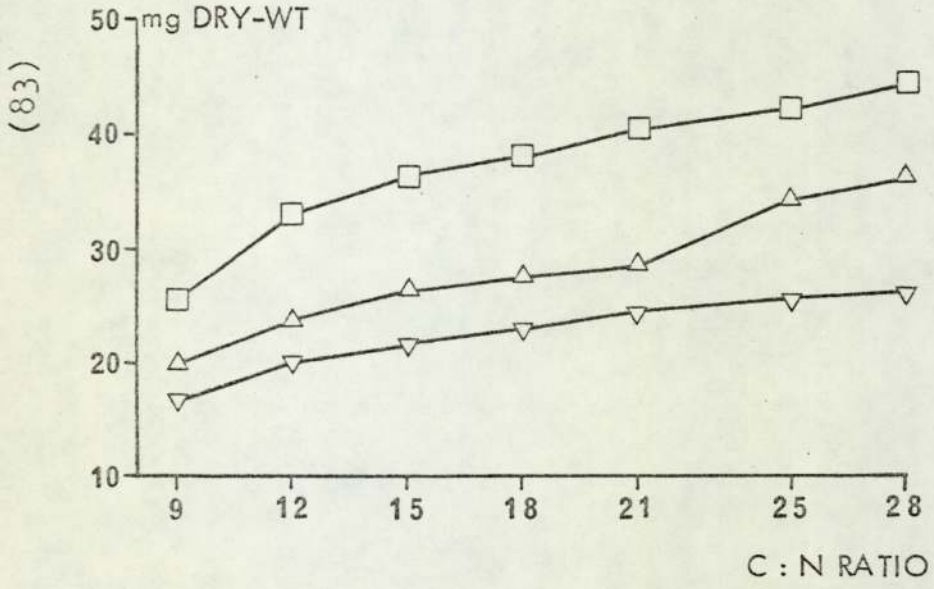
28 DAYS INCUBATION

KEY TO FIGURES 6.1 & 6.2

	PER CENT ASPARAGINE	NITROGEN CONCENTRATION (g/L)
□	0·3	0·56
△	0·2	0·372
◇	0·1	0·186
▽	0·05	0·093

Calocybe gambosa 2

YIELD



Calocybe gambosa 2

pH CULTURE FLUID

POST-INCUBATION

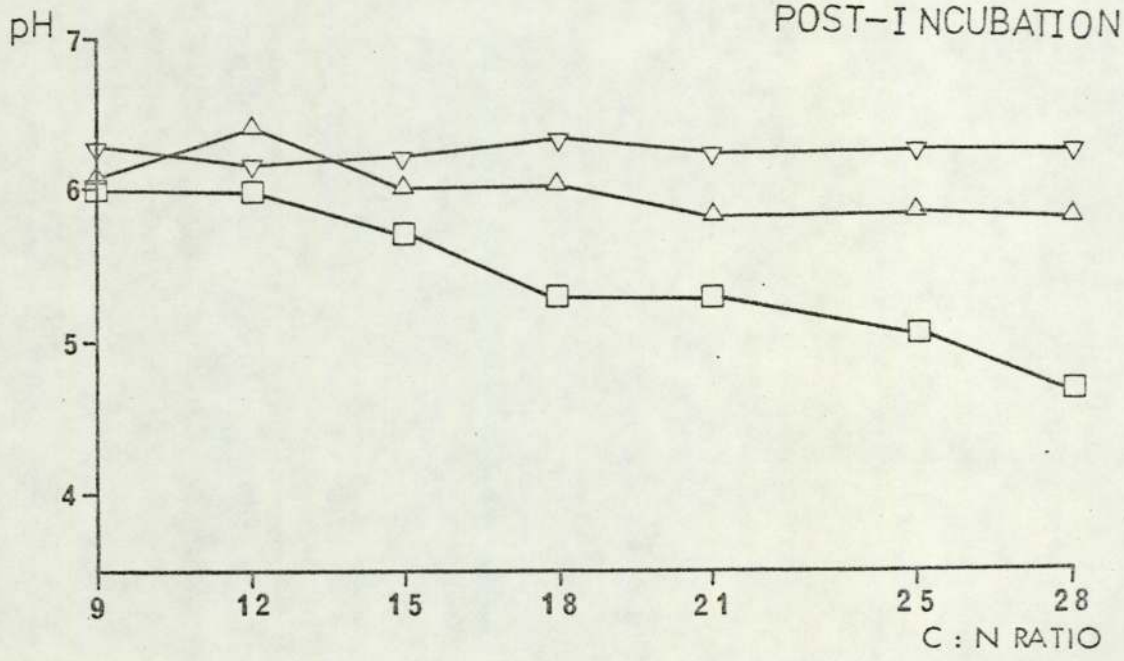
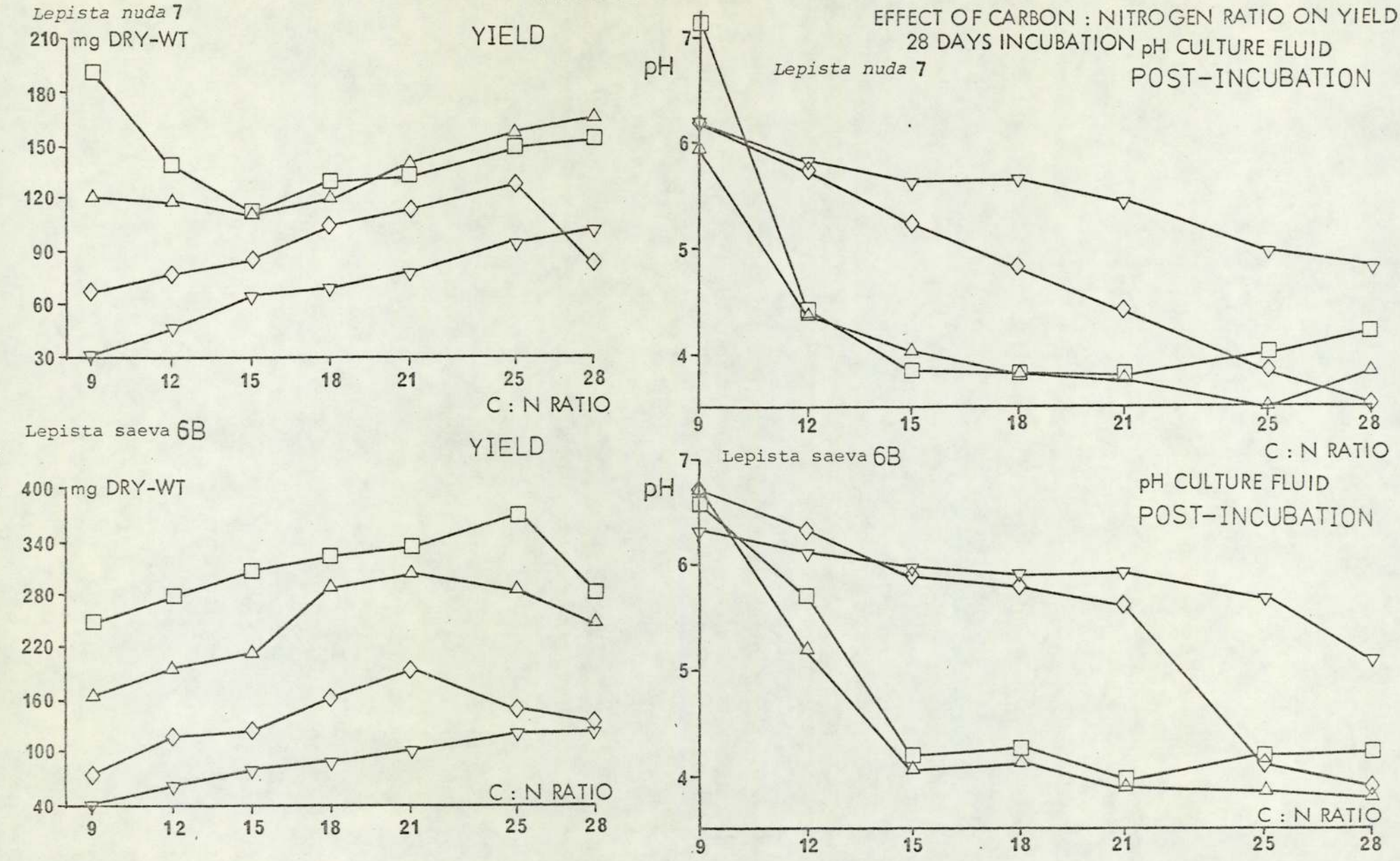


FIGURE 6.2

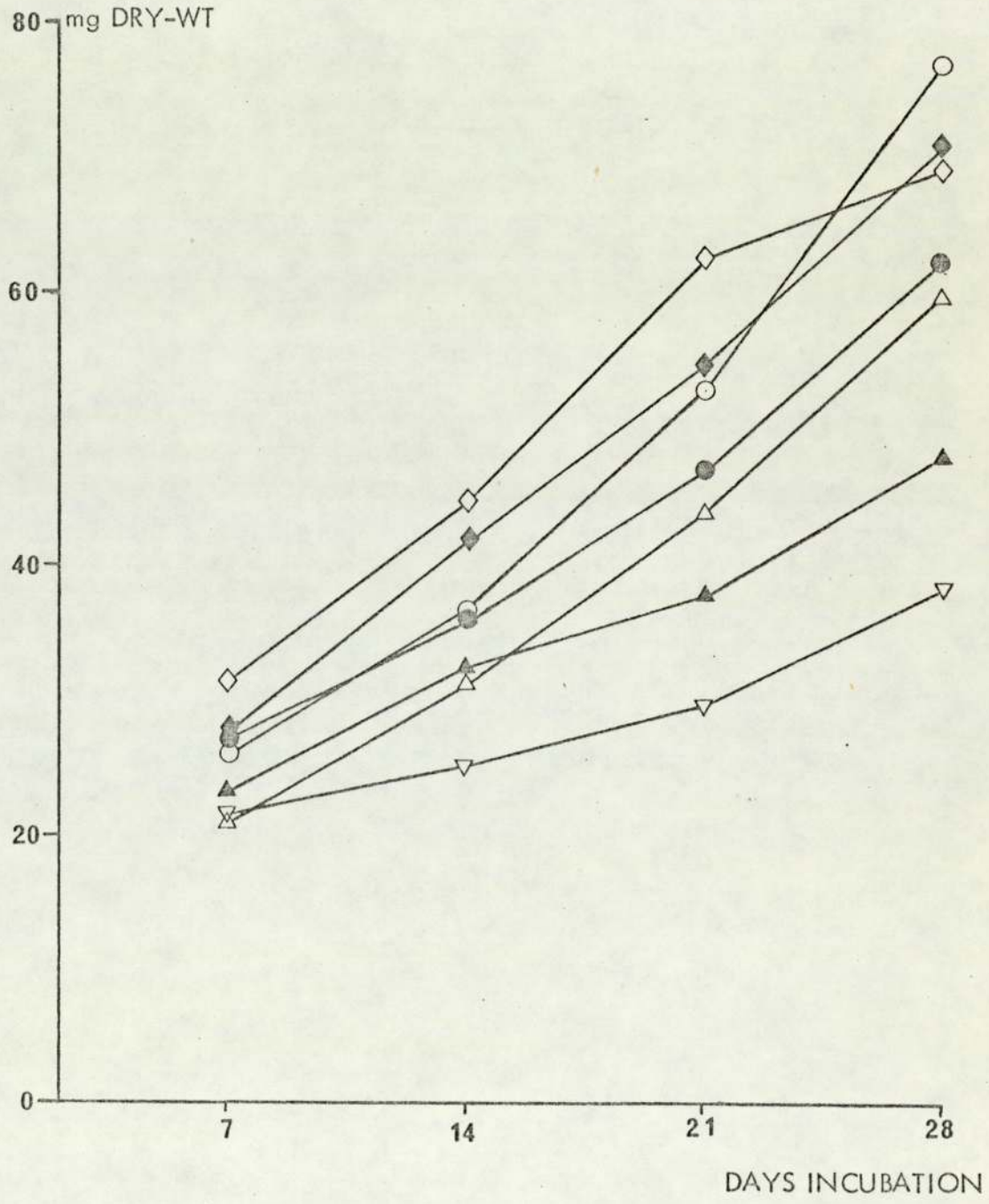

Lepista nuda 7
YIELD
mg DRY-WT
210
180
150
120
90
60
30
9
12
15
18
21
25
28
C : N RATIO
EFFECT OF CARBON : NITROGEN RATIO ON YIELD
28 DAYS INCUBATION pH CULTURE FLUID
Lepista nuda 7
POST-INCUBATION
pH
7
6
5
4
C : N RATIO
Lepista saeva 6B
YIELD
mg DRY-WT
400
340
280
220
160
100
40
C : N RATIO
Lepista saeva 6B
pH CULTURE FLUID
POST-INCUBATION

EFFECT OF CARBON : NITROGEN RATIO ON YIELD

FIGURE 6.3

0.186 g NITROGEN PER LITRE

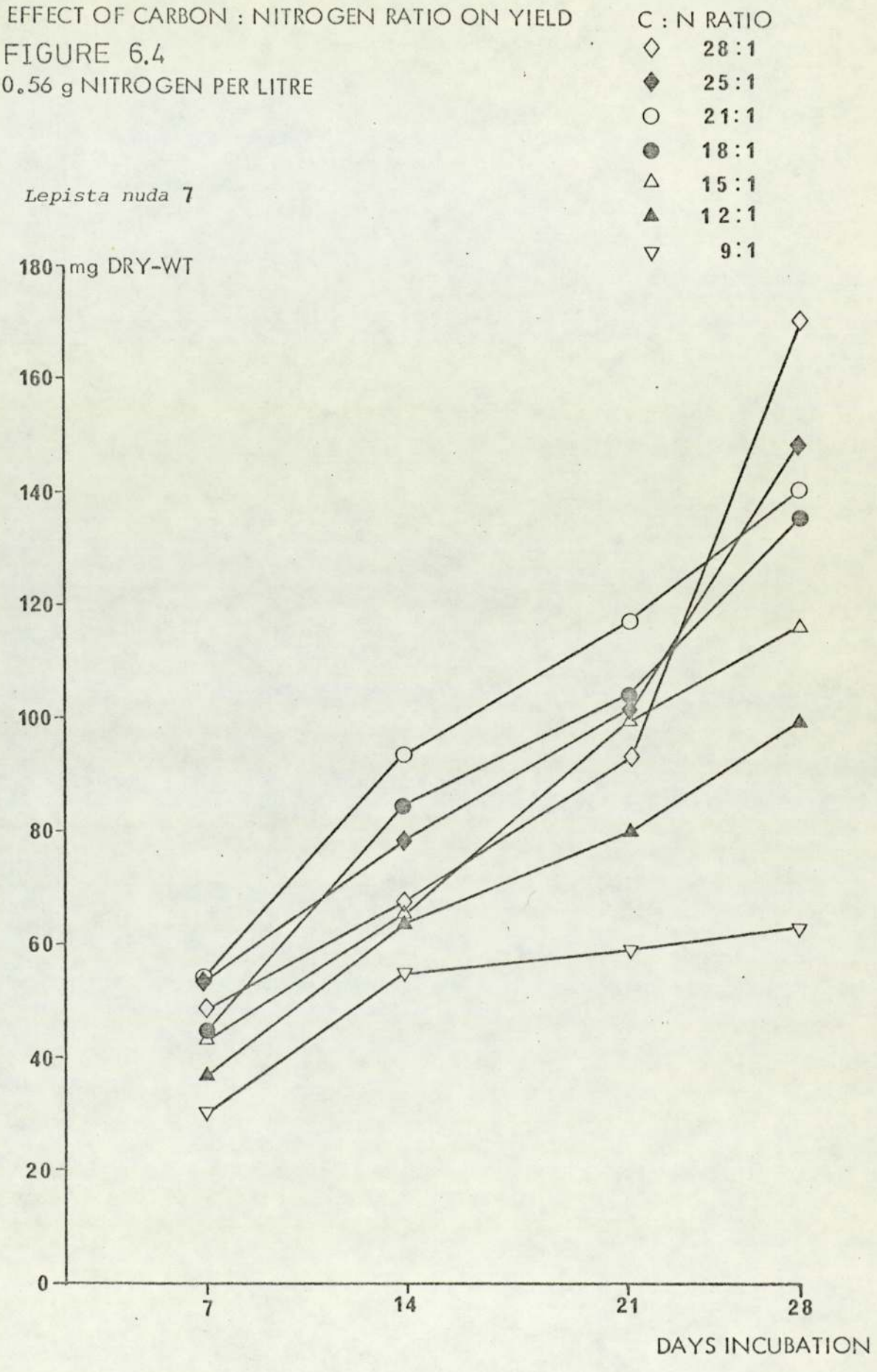
Lepista nuda 7



EFFECT OF CARBON : NITROGEN RATIO ON YIELD

FIGURE 6.4

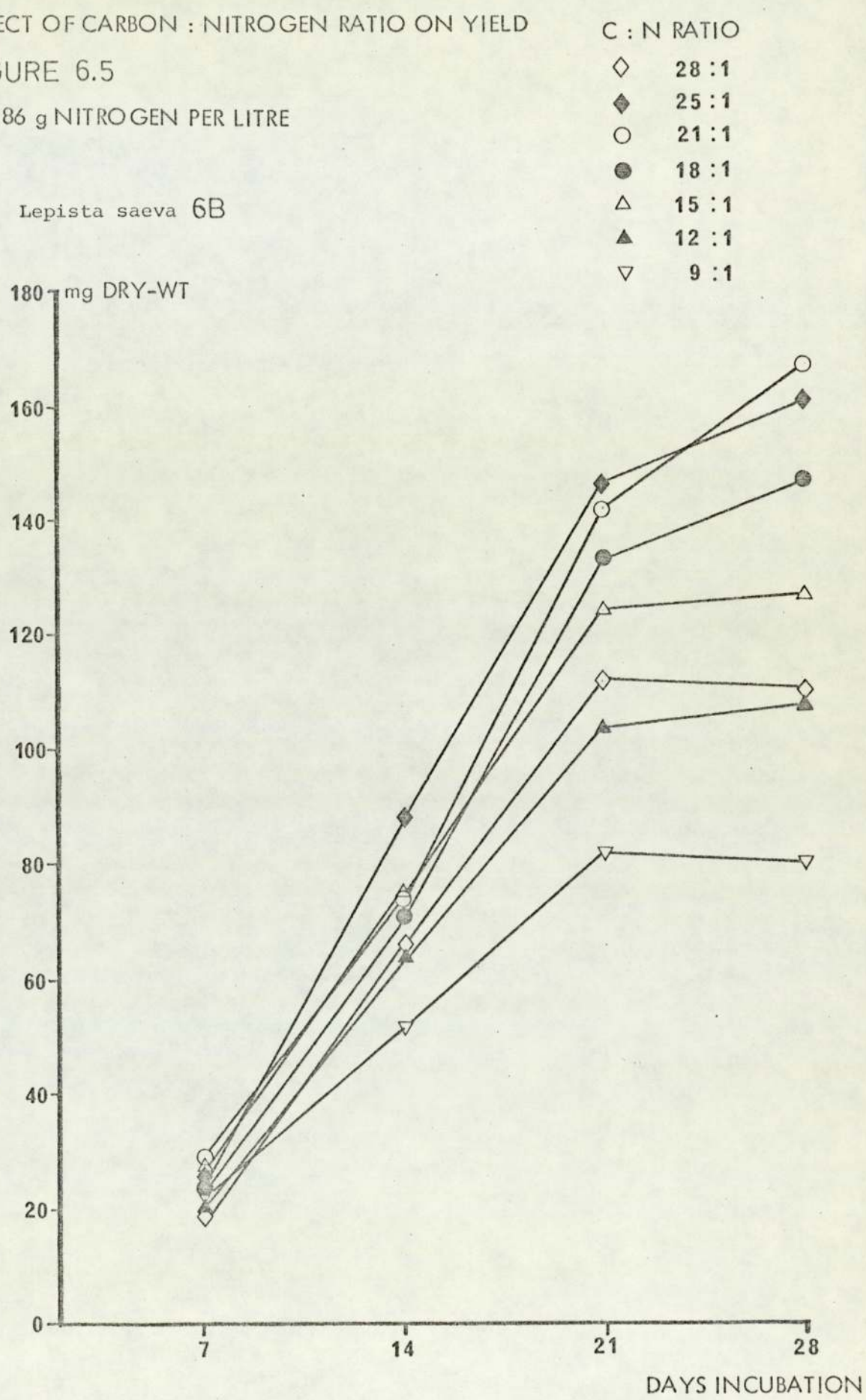
0.56 g NITROGEN PER LITRE



EFFECT OF CARBON : NITROGEN RATIO ON YIELD

FIGURE 6.5

0.186 g NITROGEN PER LITRE



EFFECT OF CARBON : NITROGEN RATIO ON YIELD

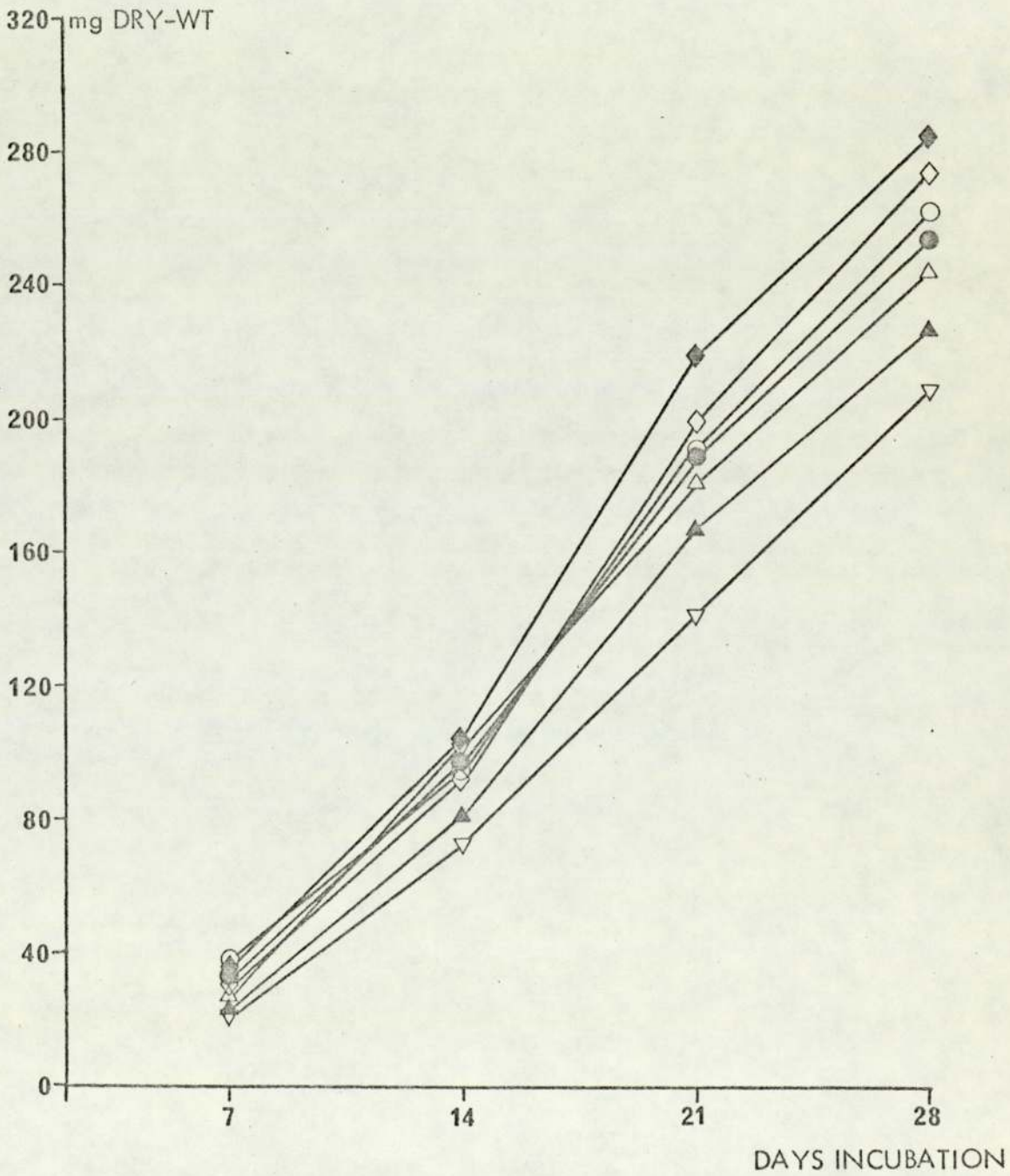
FIGURE 6.6

0.56 g NITROGEN PER LITRE

C : N RATIO

◇ 28 : 1
◆ 25 : 1
○ 21 : 1
● 18 : 1
△ 15 : 1
▲ 12 : 1
▽ 9 : 1

Lepista saeva 6B



c. 50% carbon) at 21 days, with concurrent reduction in yields at 28 days, also with nitrogen at 28 days becoming limiting (assumption of mycelial nitrogen content at c. 5%). The C/N ratio of 21:1 produced highest yields at 28 days.

For the 0.3% asparagine series (0.5592g %N) (Figure 6.6) the C/N ratio of 25:1 produced highest yields throughout. At the highest yields (284 mg) nitrogen started to become limiting. At the 9:1 ratio with the yields attained (208.6 mg) carbon level became limiting, thoughout the remaining ratios carbon and nitrogen were not limiting factors. Overall the L. saeva isolate demonstrated an ability to grow at a sustained high rate in media becoming increasingly deficient in carbon and nitrogen, including an ability to reduce its mycelial nitrogen content below that of 5%.

Average yields of both Lepista species were approximately doubled at 0.5592g %N compared to the 0.1864g %N series, these series represent two extremes, carbon limitation of the L. saeva isolate occurred in the 0.1864g %N series, together with some nitrogen limitation. In the 0.5592g %N series both carbon and nitrogen were generally present in excess, also pH shift in concentrated media at high C/N ratios is difficult to control, therefore, for the purposes of further studies the compromise of 0.3728g %N (2g Asparagine per litre) was chosen at a C/N ratio of c.22:1 (a glucose content of 20g per litre).

7:0 UTILISATION OF A RANGE OF SINGLE AND COMBINED CARBON SOURCES

The polysaccharide, tri - and disaccharides dextrin, raffinose, maltose, sucrose and trehalose produced higher yields than glucose with L. nuda isolate 7 (Figure 7.1). The polysaccharides glycogen, starch, inulin, the disaccharide cellobiose and the monosaccharides mannose and fructose also enabled high dry weights to be achieved.

Growth of the L. saeva isolate 6B was best on glucose (Figure 7.2). Mannose, dextrin, sucrose, fructose and starch, dry-weights decreasing respectively were excellent sources of carbon following glucose. The polysaccharides inulin, glycogen and xylan

FIGURE 7.1
UTILISATION OF CARBON SOURCES

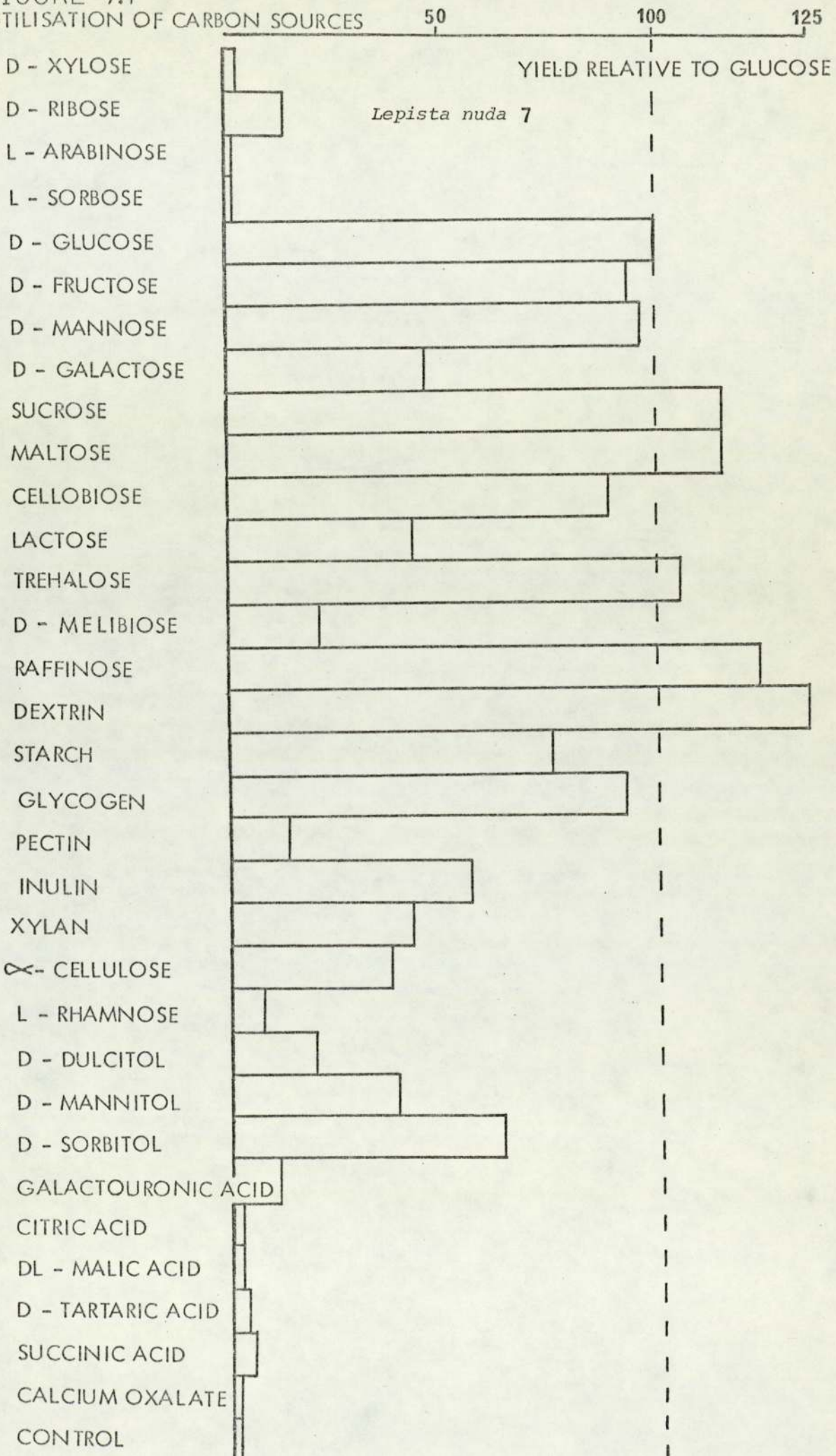


FIGURE 7.2
UTILISATION OF CARBON SOURCES

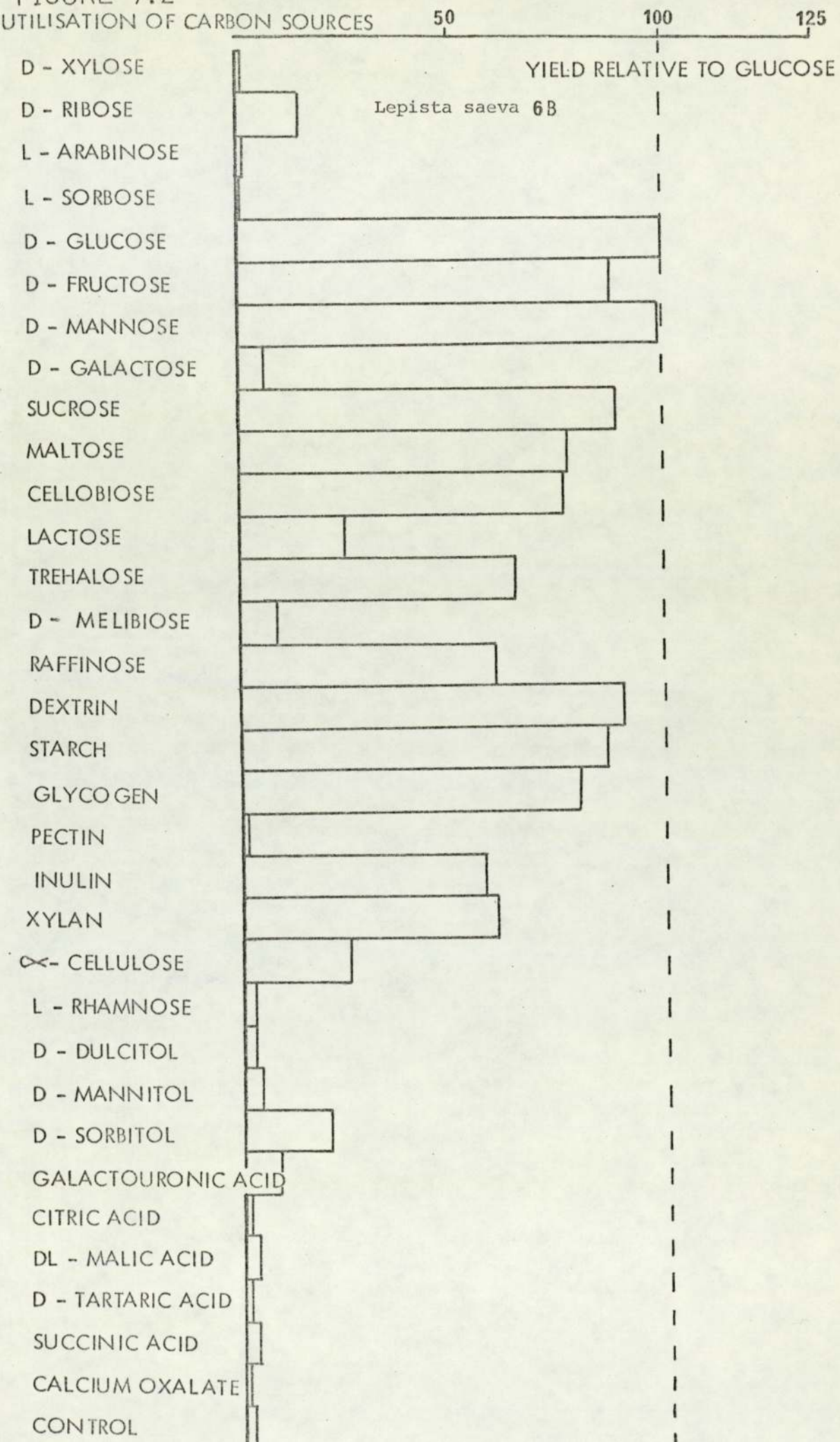


FIGURE 7.3

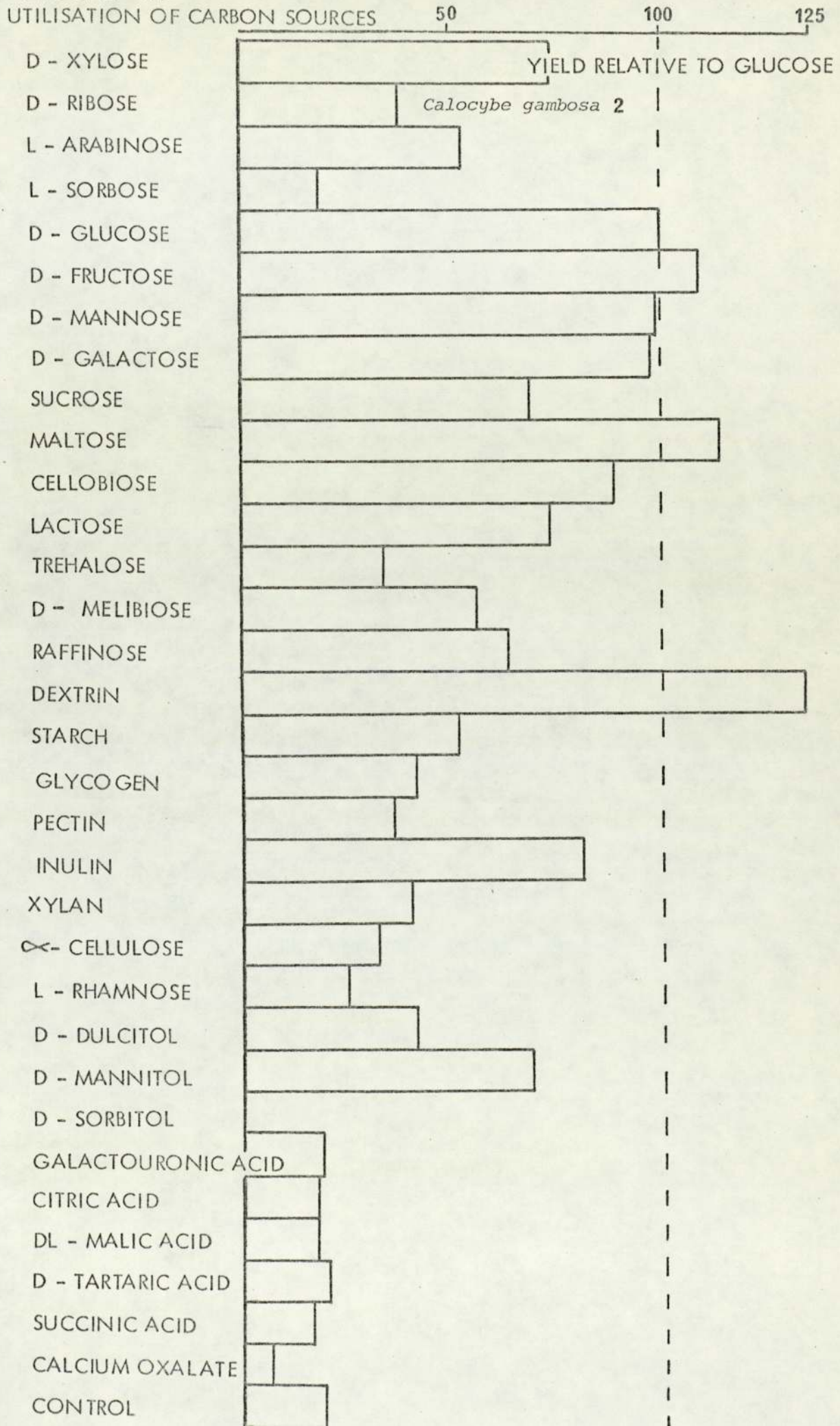

UTILISATION OF CARBON SOURCES
50
100
125
YIELD RELATIVE TO GLUCOSE
Calocybe gambosa 2
D - XYLOSE
D - RIBOSE
L - ARABINOSE
L - SORBOSE
D - GLUCOSE
D - FRUCTOSE
D - MANNOSE
D - GALACTOSE
SUCROSE
MALTOSE
CELLOBIOSE
LACTOSE
TREHALOSE
D - MELIBIOSE
RAFFINOSE
DEXTRIN
STARCH
GLYCOGEN
PECTIN
INULIN
XYLAN
CELLULOSE
L - RHAMNOSE
D - DULCITOL
D - MANNITOL
D - SORBITOL
GALACTOURONIC ACID
CITRIC ACID
DL - MALIC ACID
D - TARTARIC ACID
SUCCINIC ACID
CALCIUM OXALATE
CONTROL

TABLE 7.1 GROWTH ON COMBINED CARBON SOURCES
(COMPARED WITH D-GLUCOSE)

CARBON SOURCE	Lepista nuda 7	Lepista saeva 6B	Calocybe gambosa 2
	mg	mg	mg
D - GLUCOSE	212.7	345.0	18.2
STARCH + MALTOSE + GLUCOSE	224.7	333.0	21.8
PECTIN + GALACTOSE + GLUCOSE	150.7	44.2	11.8
INULIN + FRUCTOSE + GLUCOSE	213.4	313.6	13.4
DEXTRIN + MALTOSE + GLUCOSE	230.2	275.7	20.0
MALTOSE + FRUCTOSE + GLUCOSE	252.9	308.5	18.2
SUCROSE + FRUCTOSE + GLUCOSE	195.1	358.7	16.2
GALACTOSE + FRUCTOSE + GLUCOSE	207.1	308.3	16.9
RIBOSE + ARABINOSE + XYLOSE	3.2	1.7	16.5
CELLOBIOSE + MALTOSE + SUCROSE	235.8		10.8
MANNOSE + SORBOSE + ARABINOSE	98.6	96.2	12.2
GLUCOSE + FRUCTOSE, 1,6 DIPHOSPHATE	193.5	277.2	17.0
CONTROL	3.6	4.4	3.3

were also high yielding carbon sources, the trisaccharide raffinose and the disaccharidetrehalose, a common fungal reserve carbohydrate, supported good yields.

Growth of L. saeva on pectin was negligible, poor growth was also demonstrated by L. nuda, both achieved a low yield with the monomer galactouronic acid as carbon source. Both isolates produced moderate to low yields with cellulose, cellobiose - the terminal disaccharide(β (1-4) linked glucose) cleavage product of cellulose - served as an excellent source of carbon for each isolate. Growth was respectively better with galactose than ribose and vice versa for the L. nuda and L. saeva isolates.

Both species produced moderate yields on the hexitols sorbitol, mannitol and dulcitol. The pentose sugars, xylose and arabinose, and the keto-hexose sugar, sorbose, allowed only slight growth. The organic acids, citric, malic, tartaric and succinic together with calcium oxalate were *very* poor sources of carbon for both species. As atypical carbon sources, the disaccharide lactose, enabled moderate to poor yields, the trisaccharide melibiose and the methyl pentose sugar L-rhamnose poor growth with both isolates.

Combined sources of carbon produced yields, in general in slight excess of the yields, calculable for the corresponding amounts of the individual sugars (Table 7.1). The combination of starch, maltose and glucose was an excellent source of carbon for both species. Combination of carbon sources did not, however, markedly improve pectin, ribose, arabinose, xylose or sorbose as sources of carbon for the maintainance of mycelial growth and respiration.

The Calocybe gambosa isolate produced best growth on dextrin, maltose, fructose, glucose and mannose as the five highest yielding carbon sources (Figure 7.3). Good growth was produced on ribose, arabinose and xylose in combination and xylose and arabinose singly. Galactose and cellobiose produced good dry weights, also mannitol served as a *very* favourable carbon source.

The combination of starch, maltose and glucose was

excellent as a combined carbon source, producing the highest yield for this isolate in the series.

The organic acids, succinate, tartrate, malate and citrate plus calcium oxalate did not enable any significant growth by this species.

8:0 UTILISATION OF A RANGE OF ORGANIC AND INORGANIC NITROGEN SOURCES

Highest yields for all isolates (exception L. nuda 7) were produced on casein hydrolysate (Figures 8.1, 8.2 and 8.3). Asparagine was the best single source of nitrogen for all isolates with the exception of C. gambosa strain 2, which developed only very poorly on this substrate.

Growth on peptone was generally good for all species this was an excellent source of nitrogen for L. nuda isolates (approx., two-thirds to three-quarters that attained on asparagine), however, this was not a favourable nitrogen source for L. saeva strains (less than half yield attained on asparagine) and only a fair to poor source of nitrogen for C. gambosa isolates. Ammonium bicarbonate and urea proved to be good sources of nitrogen in general, the exceptions were poor growth on both by C. gambosa strain 1 and low yields on the latter by L. saeva strain 6B.

Growth on ammonium, sodium and potassium nitrate was highly variable, lower yields on the former was probably due to a sharp drop in pH values to inhibitory levels following utilisation of the ammonium component. Sodium and potassium nitrates were very good sources nitrogen for L. nuda isolate 7 and L. saeva isolate 5, but only a fair nitrogen source for L. nuda isolate 8 and a poor nitrogen source for L. saeva 6B. C. gambosa strain 1 grew well on ammonium nitrate, moderately with potassium nitrate and poorly on sodium nitrate, these were all poor nitrogen sources for isolate 2. Sodium nitrite proved toxic for all species compared with the control. Propionamide produced variable yields, but was in general only a fair source of nitrogen.

FIGURE 8.1

UTILISATION OF ORGANIC, AMMONIUM, NITRATE AND NITRITE NITROGEN

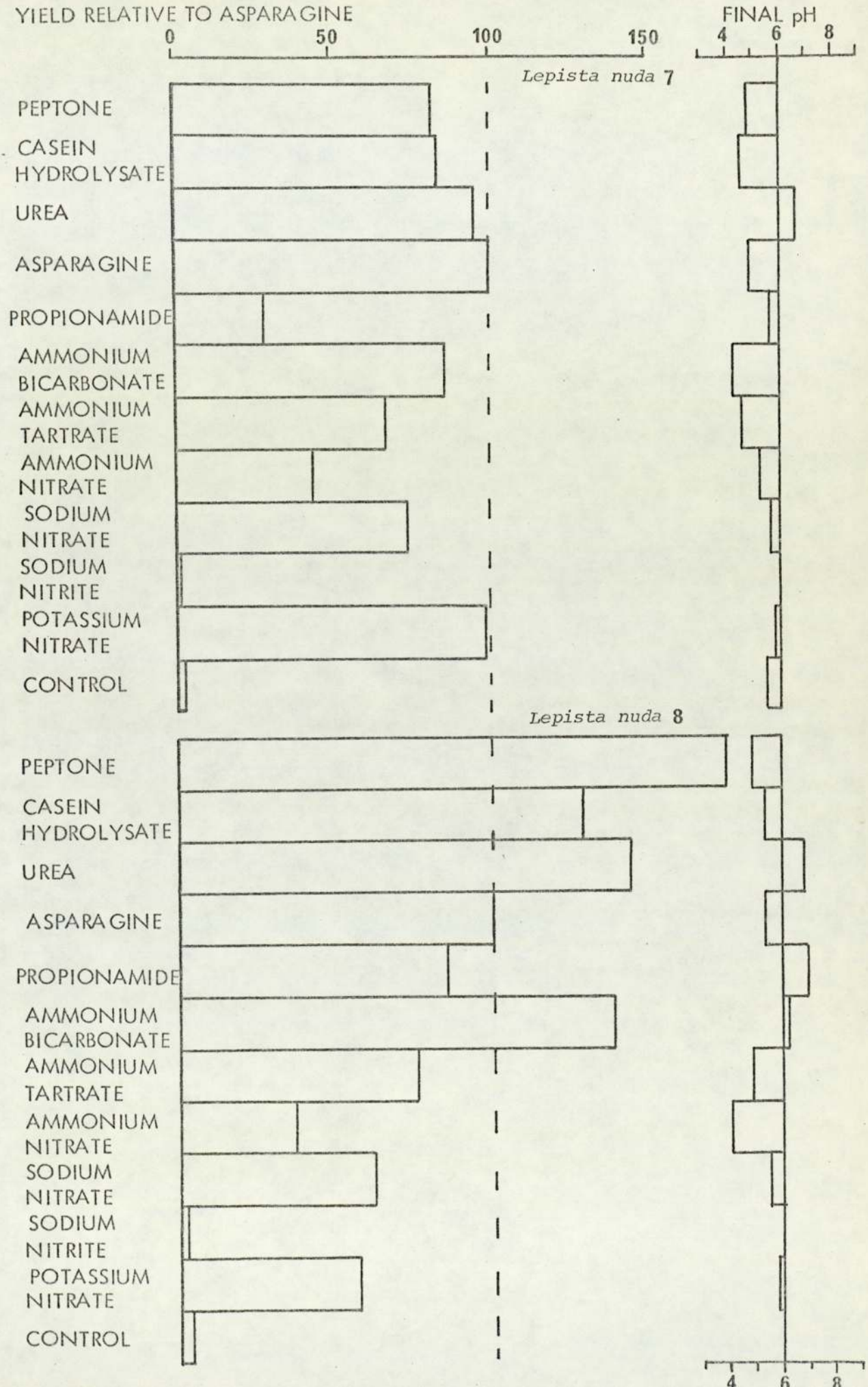


FIGURE 8.2

UTILISATION OF ORGANIC, AMMONIUM, NITRATE AND NITRITE NITROGEN

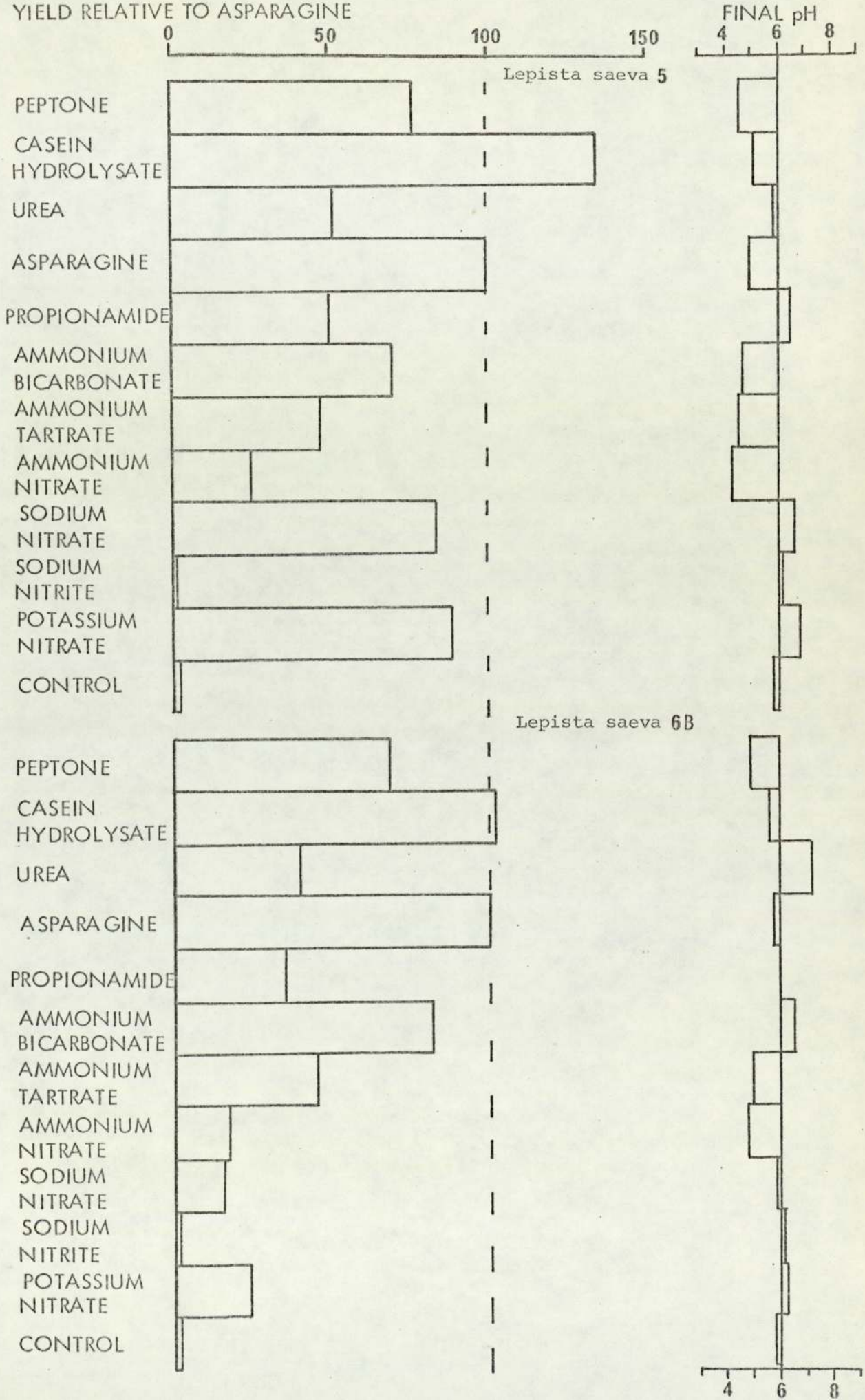
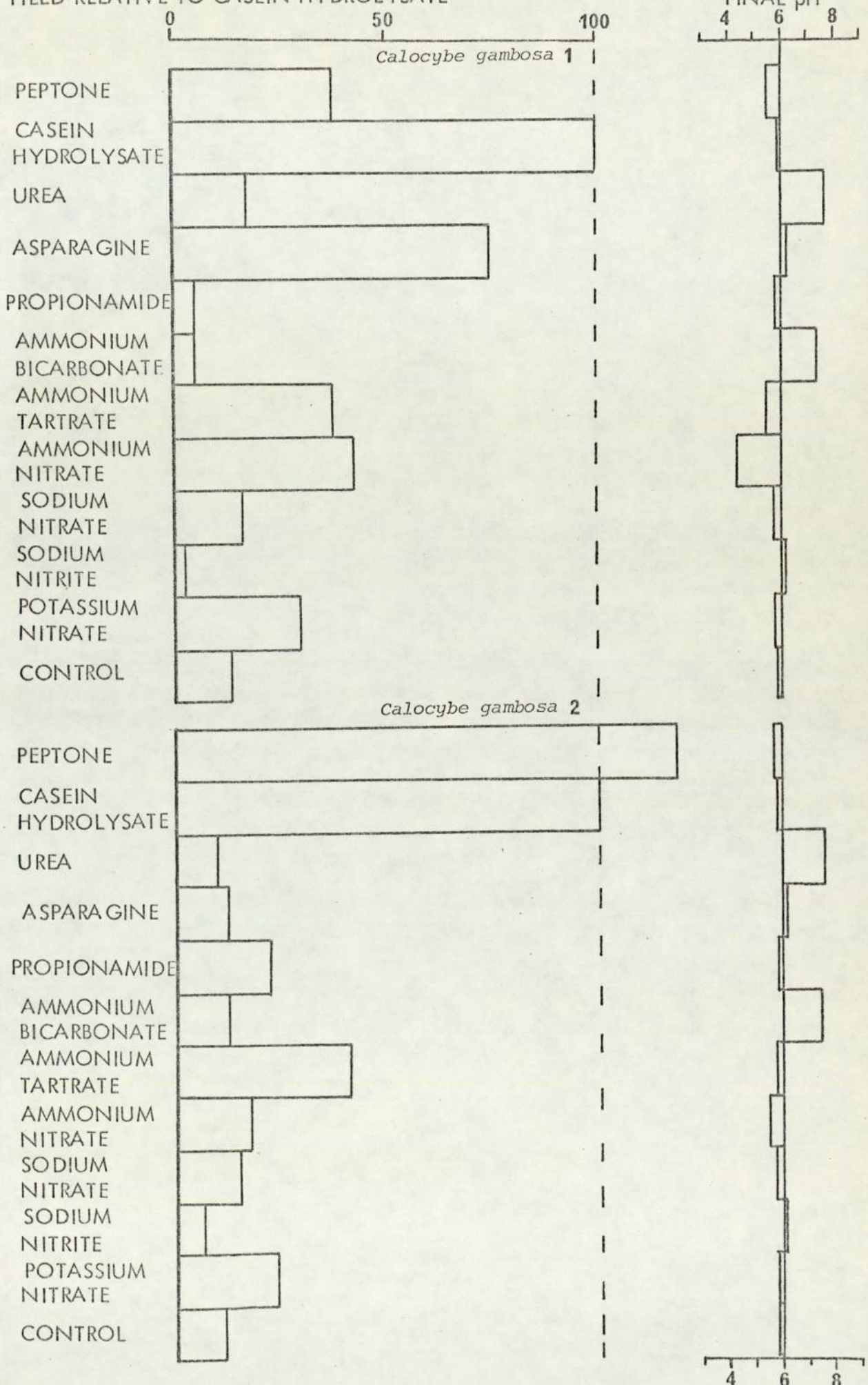


FIGURE 8.3

UTILISATION OF ORGANIC, AMMONIUM, NITRATE AND NITRITE NITROGEN

YIELD RELATIVE TO CASEIN HYDROLYSATE



Change in the hydrogen ion concentration with metabolism of these substrates affected yields where this exceeded the buffer capacity. The most marked reduction in pH values occurred with ammonium nitrate and ammonium tartrate. A shift to higher pH values was produced by growth of L. saeva isolate 5 on potassium and sodium nitrates and also with isolate 6B on the former.

Growth on urea produced alkaline culture fluids following incubation, this was particularly marked in the case of the C. gambosa isolates, a shift to higher pH values also occurred with this species on ammonium bicarbonate and asparagine. This pH trend may reflect the net release of NH_3 into the culture fluid by metabolism of these substrates, possibly having a direct staling effect which rapidly inhibited further growth.

Propionamide and ammonium bicarbonate also caused a shift to higher pH values, than the control, in the culture fluids of the individual isolates of L. nuda and L. saeva 6B, which may reflect liberation of ammonia following metabolism of these chemicals.

8:1 Utilisation of a Range of Amino-acids as Single and Combined Sources of Nitrogen

Aspartic acid, leucine, glycine, alanine, glutamic acid, serine and arginine were, in order of declining yield, the best amino-acids as sole nitrogen sources for growth of the L. nuda isolate (7) (Figure 8.4). Aspartic acid and leucine produced favourable yields, both the amino-acid mix and casein hydrolysate were very good compound sources of nitrogen. Fair yields, approximately half that attained with asparagine were produced with threonine and methionine.

The L. saeva strain(6B) produced highest dry-weights declining respectively, with serine, glycine, glutamic acid, aspartic acid and alanine (Figures 8.5). The amide glutamine was only a poor source of nitrogen. Leucine and arginine supported low yields. Casein hydrolysate was an excellent compound nitrogen source, however, the amino-acid mix gave only low yields indicating an inhibitory influence

FIGURE 8.4
UTILISATION OF AMINO-ACID AND AMIDE NITROGEN SOURCES

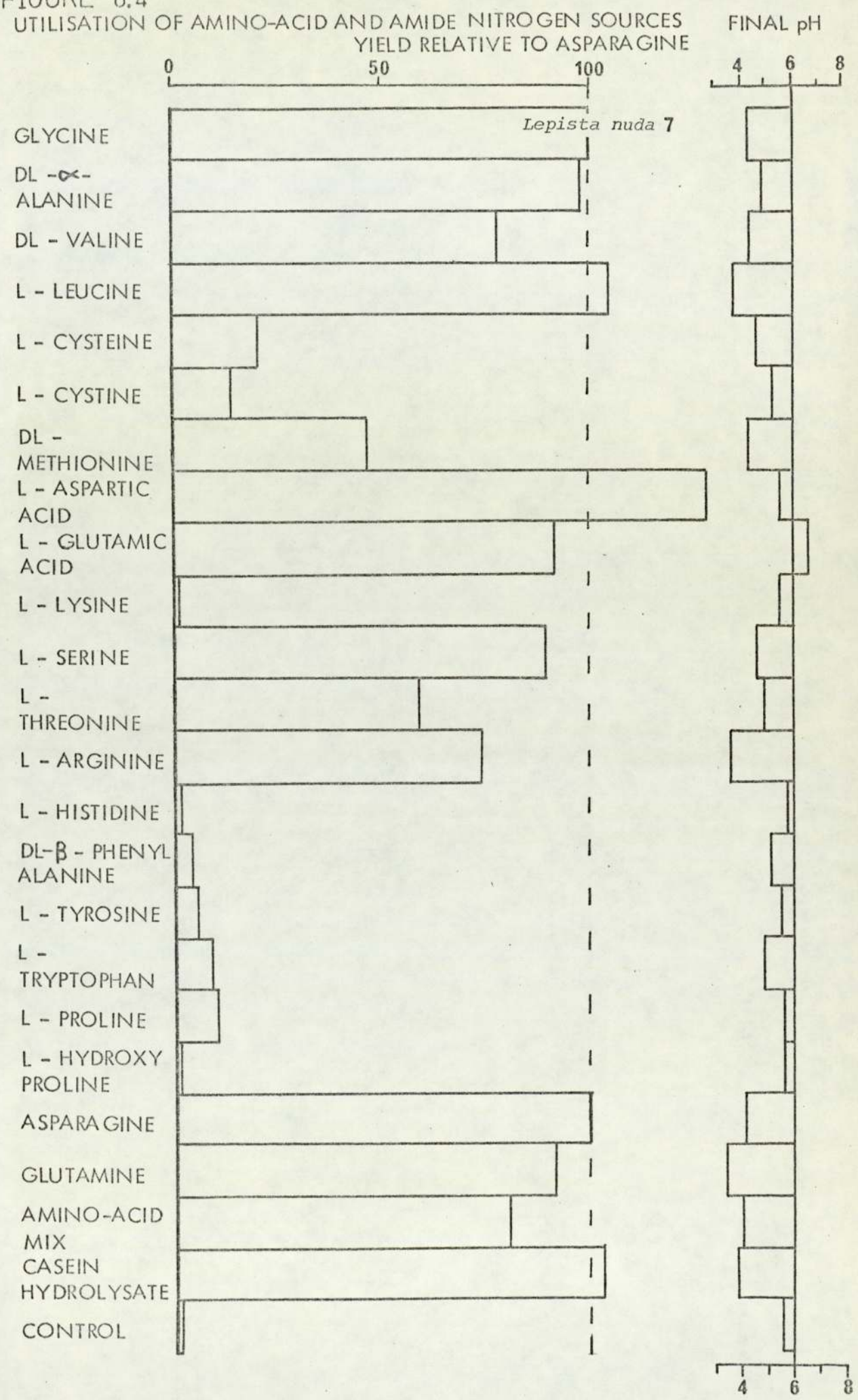


FIGURE 8.5
UTILISATION OF AMINO-ACID AND AMIDE NITROGEN SOURCES

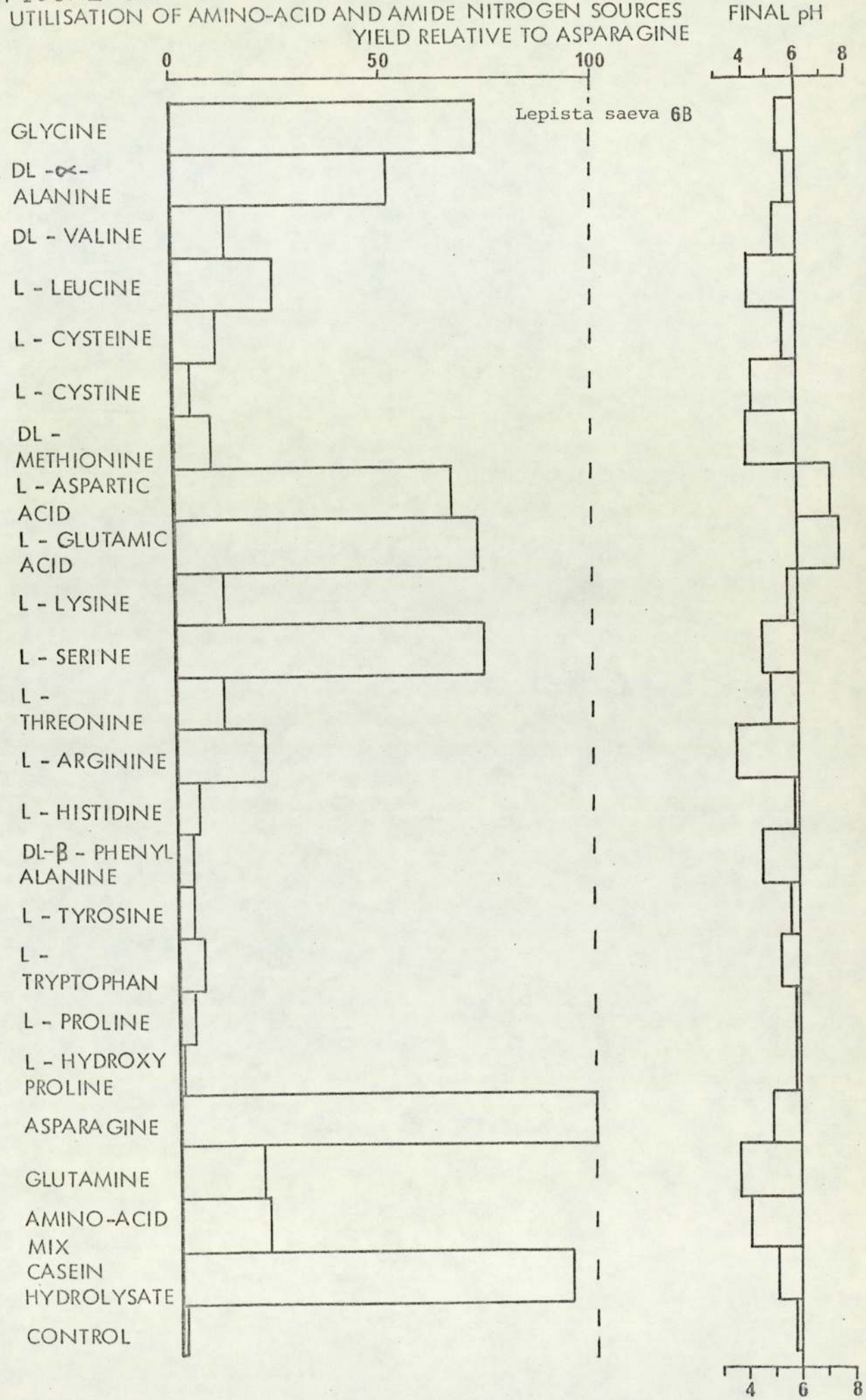


FIGURE 8.6

UTILISATION OF AMINO-ACID AND AMIDE NITROGEN SOURCES

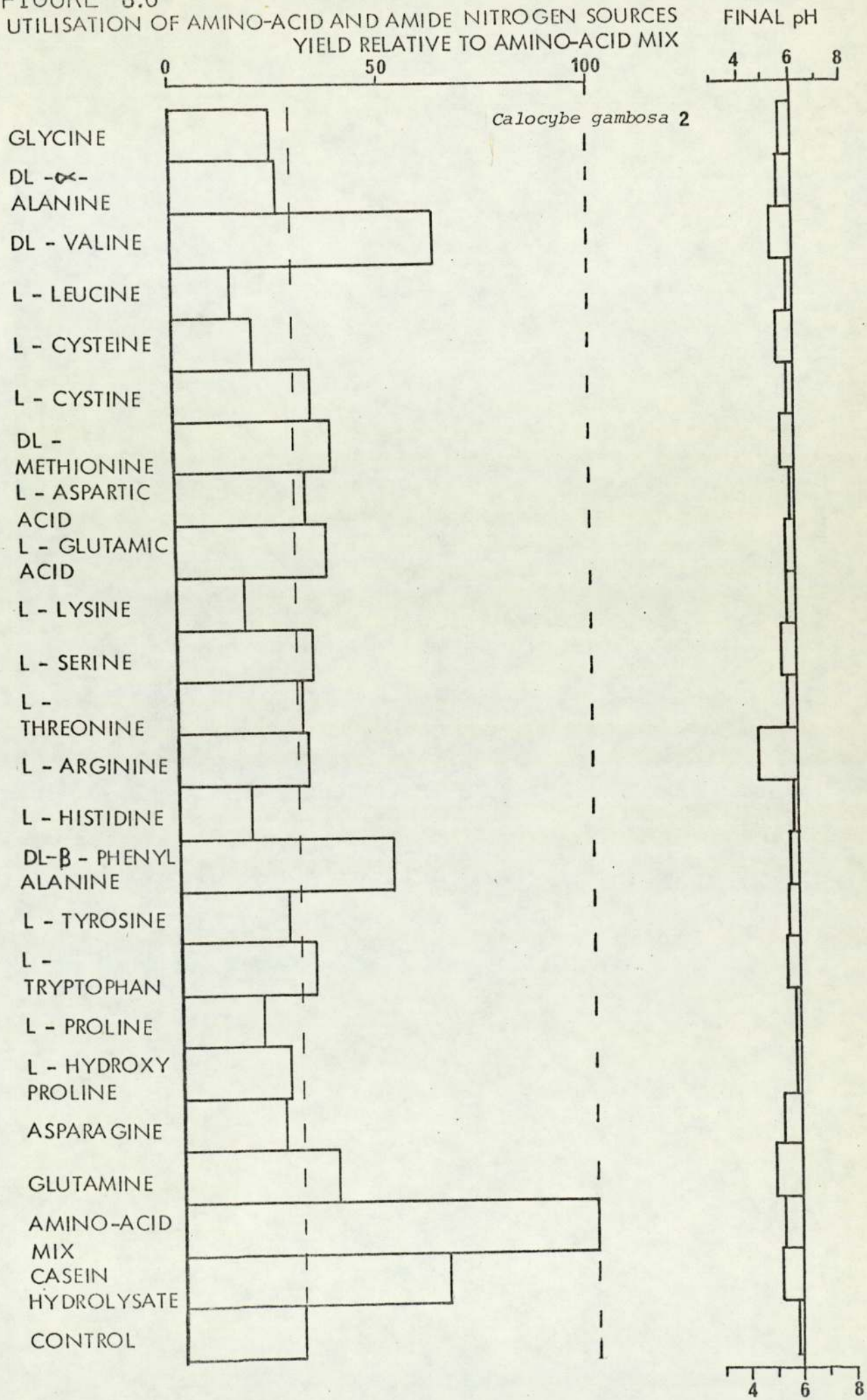


TABLE 8.1

VALUES FOR RESIDUAL GLUCOSE FOLLOWING INCUBATION OF L. SAEVA 6B ON SIX SOURCES OF AMINO-ACID NITROGEN.

NITROGEN SOURCES	RESIDUAL GLUCOSE (mg)
INITIAL	500
GLYCINE	135
SERINE	148
ASPARAGINE	42.5
GLUTAMINE	308.2
AMINO-ACID MIX	280
CASEIN HYDROLYSATE	82.5

Results determined for a single flask averaged from the pooled culture fluids of 3 replicate flasks by the 3, 5, dinitrosalcyclic acid method (Lindsay 1973).

possibly due to larger amounts of certain inhibitory amino-acids in this combination.

Good growth of the C. gambosa strain was produced with the amino-acid mix, less satisfactory growth on casein hydrolysate (Figure 8.6). The single amino-acids valine and phenylalanine produced highest yields. Methionine and glutamic acid plus its amide glutamine produced yields in slight excess of that given by the control.

Growth of L. nuda and L. saeva isolates showed a marked drop in the pH of the culture fluids following growth on arginine, leucine and methionine plus glutamine and the amino-acid mix, a slightly less marked shift in pH occurred following growth on glycine, valine, phenylalanine and cysteine.

Aspartic and glutamic acid produced net alkaline conditions for the L. saeva isolate, the latter caused a shift to neutrality with the L. nuda strain. Growth on casein hydrolysate produced a significant pH drop in the culture fluid of the L. saeva isolate.

The C. gambosa isolate produced a marked change to acid values in the culture fluid following growth on arginine.

The culture fluid of the L. saeva isolate was dark black following growth on tyrosine and the amino-acid mix, and brown with growth on casein hydrolysate, a black colouration of the medium was produced by the L. nuda isolate on tyrosine, the C. gambosa isolate produced black submerged myceliumwith tyrosine, indicating release of melanin pigments or intermediates.

A dark yellow culture fluid was produced by the L. saeva isolate on glycine, arginine and asparagine and a pink colouration on proline. L. nuda (7) also produced a dark yellow discoloured medium on arginine.

The culture fluid of the C. gambosa isolate was bright orange-yellow except following growth on cysteine, cystine, lysine, histidine, phenylalanine, tyrosine, proline and hydroxyproline.

The L. nuda and L. saeva isolates produced significant amounts of a polysaccharide type material on the underside of the mycelial mat following growth on certain of the amino-acids. Accumulation of this material was heaviest following growth on glutamine. Medium production occurred on arginine with both species and singly by the L. nuda isolate on glycine, valine, cystine, with light accumulation by this species on alanine, glutamic acid, amino-acid mix and casein hydrolysate as sources of nitrogen.

Values for residual glucose following growth of the L. saeva isolate (Table 8.1) which attained a highest yield of 335.6 mg on asparagine indicate approximately nine-tenths utilisation of substrate glucose; utilisation of glucose was roughly proportional to final dry-weight.

The figures indicate that the glucose asparagine basal medium adapted in these studies was capable of supporting high yields and did not become at twenty-eight days incubation carbon or nitrogen limited.

SECTION 4

9:0 AFFECT OF ETHANOL, SIMPLE ORGANIC ACIDS, AND FATTY ACIDS AS MEDIUM SUPPLEMENTS

Both L. nuda and L. saeva isolates did not show significant ($P = 0.05$) growth promotion with these medium supplements (Table 9.1). The optimum concentration for addition of the organic acids was critical. Concentrations in excess of 100 mg/L strongly inhibited growth, additions of these chemicals at higher levels made pH difficult to control. A fall in yield was also marked with the higher levels of addition of oleic, linoleic and palmitic (L. nuda isolate) acids where growth was assessed in stationary liquid culture.

Acetate, oleic, linoleic, stearic and palmitic acids caused a considerable increase in density of colony growth, clearly

TABLE 9.1

ADDITION (ORGANIC ACID, ALDEHYDE, ALCOHOL)	CONCENTRATION mg/L	LEPISTA NUDA 7 mg	LEPISTA SAEVA 6B mg	CALOCYBE GAMBOSA 2 mg
Acetate (Acetic Acid)	50	144.2	189.6	7.2
	100	135.6	189.9	19.5
Acetaldehyde	50	132.6	198.2	6.5
	100	124.3	194.0	6.1
N-Propionic Acid	50	146.0	186.5	26.0
	100	151.4	176.7	9.0
Iso-Valeric Acid	50	128.6	198.4	31.7
	100	151.1	176.5	25.7
N-Butyric Acid	50	131.3	171.8	62.7
	100	137.3	172.2	12.9
Ethanol	100	125.7	198.9	10.0
	500	126.0	201.9	10.6
Oleic Acid (18:1)	100	153.1	178.0	16.6
	500	85.9	86.0	13.0
Linoleic Acid (18:2)	100	137.2	142.3	15.9
	500	72.4	49.9	4.2
Palmitic Acid (16:0)	100	158.0	154.6	12.6
	500	92.0	154.9	12.0
Stearic Acid (18:0)	100	149.3	195.6	8.7
	500	108.0	193.8	6.5
CONTROL		146.3	190.3	14.8
L.S.D. (P = 0.05)		16.8	22.1	13.5

discernable in Petri-plate studies, this was particularly so with linoleic and oleic acid additions; L. saeva produced close to tissue development with the two latter supplements. The purple pigment produced by these species tended to become concentrated into the dispersed droplets of these fatty acids.

Propionic, valeric, oleic and palmitic acid produced a non-significant elevation in yield of the L. nuda isolate. Ethanol valeric acid and acetaldehyde produced a non-significant growth increase in L. saeva, this species in Petri-plate studies showed best development with ethanol, oleic and palmitic acid additions at c. 1g/L .

High yields by C. gambosa (Table 9.1) on butyric, acetic and valeric acids were through development of sectors. Growth in Petri-plates with palmitic acid, linoleic acid and acetaldehyde supplements at c. 1g/L also caused the development of sectors.

10:0 GROWTH RESPONSE TO A RANGE OF LEAF AND MANURE EXTRACTS AS SOURCES OF GROWTH FACTORS (FIGURE 10.1)

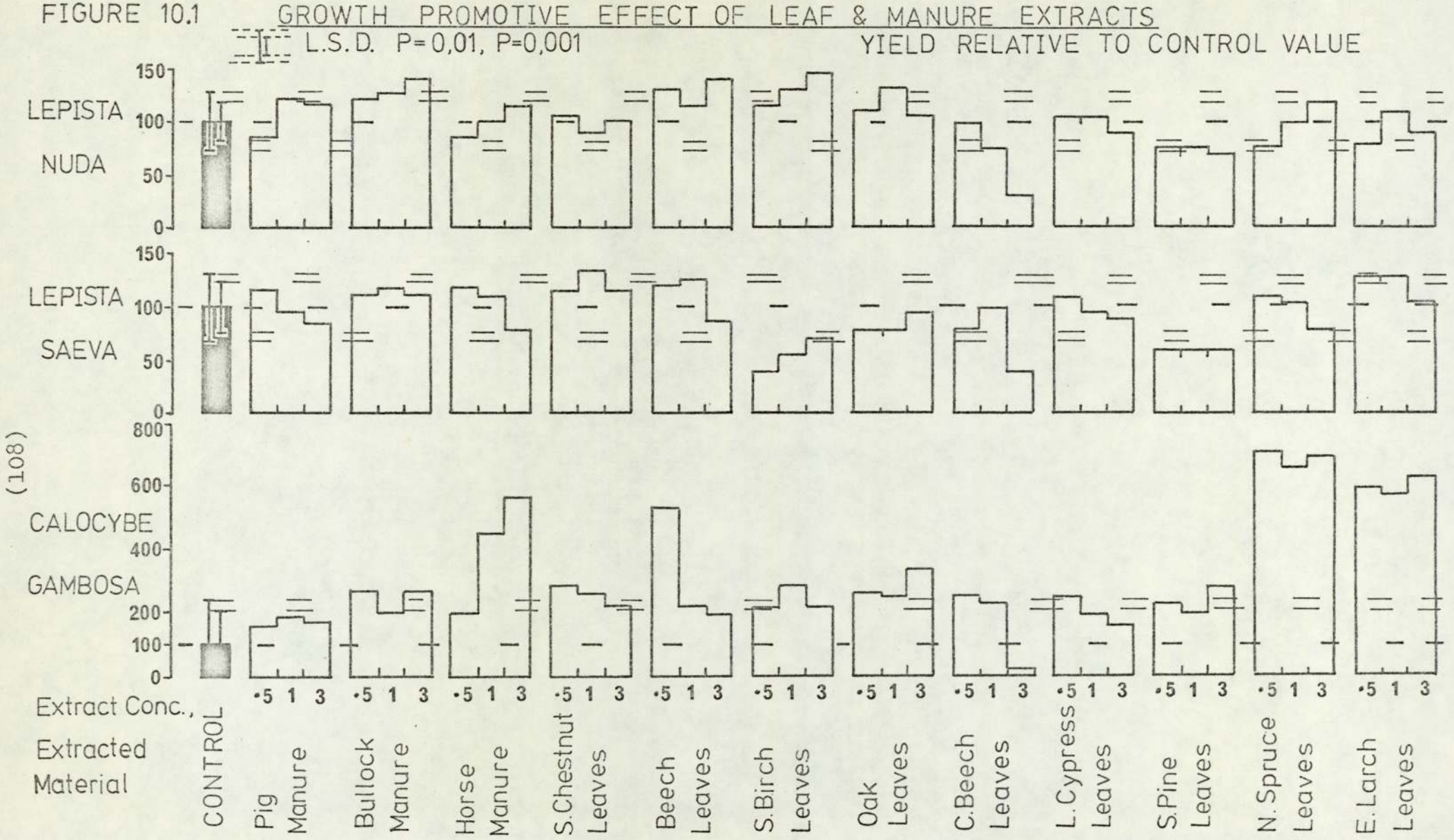
Growth by L. nuda isolate 7 was very significantly ($P = 0.001$) improved with additions of extracts from beech (0.5 &3.0 mg/L) birch (1.0 &3.0 mg/L) and oak (1.0 mg/L) together with bullock manure extract (3.0 mg/L) compared to the unsupplemented control value.

Significant ($P = 0.01$) elevation in yield occurred from addition of pig (1.0 g/L) and bullock manure (0.5 &1.0 mg/L) extracts.

L. saeva isolate 6B demonstrated a very significant growth increase only on media supplemented with sweet chestnut leaf extract, significantly improved values were obtained with beech leaf (1.0 mg/L) and larch leaf (0.5 &1.0 mg/L) extract additions .

Highly significant increases in dry-weight in relation to the control value were achieved by the C. gambosa isolate following addition of a spectrum of extracts. Larch and spruce leaf extracts were particularly potent in their ability to stimulate growth at all levels

FIGURE 10.1 GROWTH PROMOTIVE EFFECT OF LEAF & MANURE EXTRACTS



of addition. Horse manure stimulated yields in rough proportion to its level of addition, best growth with beech leaves was produced at the lowest level of addition. The leaf extracts produced from deciduous trees were broadly active, extracts from Scotch pine and Lawson's cypress were considerably less active than those from Norway spruce and European larch. Pig manure did not cause a significant growth promotion.

It is probable that growth factor(s) for C. gambosa were contained in a number of these extracts.

Copper beech at 3.0 mg/L addition was inhibitory to growth of all species.

SECTION 5

11.0 ASSESSMENT OF CELLULOLYTIC ABILITY WITHIN AND BETWEEN SPECIES

Clearing of an opaque ball-milled cellulose medium was assessed for two concentrations of cellulose in agar columns and by comparison, in relation to colony growth on agar discs in Petri-plates (Table 11.1). The former technique indicates the net cellulolytic activity of colonies where there is a physical limitation on extension growth, the latter method, which is considerably less precise, demonstrates zones of clearing beneath actively growing colonies.

The L. nuda isolates demonstrated a considerably weaker cellulolytic ability than the range of L. saeva isolates, mean values were approximately one-half those obtained by the latter. A large component of the variability in the standard error of the mean for the L. saeva strains was due to the poor cellulolytic ability of isolates C.B.S. 355.33 and 6A, the latter isolate was obtained in deciduous leaf-litter in a similar situation to that in which L. nuda frequently grows.

Clearing by L. nuda strains was generally in localised regions within the colony on Petri-plates giving rise to pear-shaped or semicircular regions where clearance was complete, but frequently mottled regions of incomplete clearing. L. saeva strain 6A also demonstrated

TABLE 11.1

SPECIES ISOLATE NO.	TUBES				PETRI - PLATE	
	0.25 per cent		1 per cent		1 per cent	
	AGAR VOLUME CLEARED (cm^3)	CELLULOSE UTILISED AS A % of* 0.0375g	AGAR VOLUME CLEARED (cm^3)	CELLULOSE UTILISED AS A % of 0.0375g*	AGAR VOLUME CLEARED (cm^3)	CELLULOSE UTILISED AS A % of 0.2g**
LEPISTA NUDA						
1	4.2	28	1.2	32	3.2	16
247.69	4.7	31	1.7	45	4.4	22
5	2.5	17	2.1	56	3.8	19
6			0.86	23	0.53	3
7	7.3	49	1.1	30	4.9	25
8	3.4	23	0.86	23	8.4	42
9	4.7	31	.86	23	1.1	6
10			1.2	32	4.9	25
11	3.0	20	1.5	40	3.8	19
		28.4 ± 4.0		33.8 ± 3.8		19.7 ± 3.8
LEPISTA SAEVA						
1	7.3	49	3.3	88	15.6	78
355.33	4.2	28	1.3	35	11.7	59
248.69					17.7	89
5	8.4	56	3.3	88	11.7	59
6A	4.7	31	1.3	35	†	
6B	12.0	80	3.7	99	11.0	55
7	15.0	100	3.0	80		
8	14.0	94	3.4	91		
		62.6 ± 11.0		73.7 ± 10.2		68.0 ± 6.6
CALOCYBE GAMBOSA						
1	5.2	35	Tr.		Tr.	
2	5.4	36	Tr.		Tr.	
		35.5				

* Weight of Cellulose in 15 ML 0.25% Cellulose Agar

** Weight of Cellulose in 20 ML 1.0% Cellulose Agar

† Clearing of Cellulose in v. Localised Regions.

Tr Trace Clearing (Indistinct Zone).

the formation of clearance zones only in restricted areas of the colony growing in Petri-plates. These characteristics are indicitive of the development of secunds.

Clearing by L. saeva strains was generally very distinct in Petri-plates, extending in a complete circular zone from the colony centre to a zone of partial clearing at the boundary of the colony, suggestive of a uniform production of cellulase throughout the colony.

Clearing of cellulose agar by the two C. gambosa isolates was apparent only at a concentration of 0.25 per cent, although clearing and some growth did occur in Petri-plates at 1 per cent, this did not extend through the depth of the agar and therefore its limits could not be clearly defined.

12:0 ASSESSMENT OF P-DIPHENOL OXIDASE LEVELS WITHIN AND BETWEEN SPECIES

The strength of the Bavendamm 'oxidase' reaction was variable within the L. nuda and L. saeva species, the C. gambosa isolates tested produced a clear indication of p-diphenol oxidase activity on both gallic and tannic acid substrates (Table 12.1).

The L. nuda strains 6, 9, 11 & 13 obtained from isolates growing in soil showed strong oxidase activity, it is possible that those strains inhabiting the lower layers of leaf-litter (at the litter-soil boundary zone) would possess higher levels of phenoloxidase activity than strains colonising recently fallen litter.

The oxidase reactions of L. saeva isolates all (except 6A) formally growing in pasture land soils did not, however, exhibit high levels.

Growth of all L. nuda and L. saeva isolates tended to be strongly or totally inhibited on the gallic acid medium, improved growth and more pronounced discolouration was shown by a number of the isolates of both species with the tannic acid medium.

Formation of a cherry-red zone beneath the colonies and inocula of the C. gambosa isolates and L. saeva strain 1 may indicate

TABLE 12.1

SPECIES	ISOLATE NO.	GALLIC ACID OXIDASE RTN	GALLIC ACID GROWTH	TANNIC ACID OXIDASE RTN	TANNIC ACID GROWTH
LEPISTA NUDA	1†	+	-	+	PI
	247.69	+	-	+	PI
	493.48	+	-		
	300.58	+	-		
	5	+	-	++	PI
	6	++	SI	++	SI
	7	(+)	-	+	SI
	8	(+)	-	+	PI
	9	++	SI	++	PI
	10	+	-	+	NI
	11	++	SI		
	12			+	NI
	13			++	PI
LEPISTA SAEVA	1†	+	-	++	SI
	248.69	+	-	+	SI
	355.33	++	NI	++	NI
	5*	++	NI	++	NI
	6A	+	PI		
	6B*	+	-	+	-
	7*			+	Tr
CALOCYBE GAMBOSA	1†	++	NI	++	NI
	2†	++	NI	++	NI

KEY

	OXIDASE REACTION		GROWTH
(+)	Discolouration Confined to Inoculum	-	No Growth
+	Discolouration in Inoculum and weakly in zone beneath	Tr	Trace growth on Inoculum
++	Strong discolouration of medium	SI	Strongly Inhibited
*	Inoculum v. strongly discoloured	PI	Partly Inhibited
†	Discolouration as a Distinct Brown-Cherry-Red zone on Tannic Acid Medium	NI	No Inhibition.

the presence of intermediate breakdown products of tannic acid; clearing of the agar beneath the fungal mat of L. saeva strain 355.33 as distinct from a discoloured peripheral zone may indicate utilisation of tannic acid or its breakdown products as carbon sources.

SECTION 6

13:0 GROWTH IN SOLID SUBSTRATES

SPECIES	ISOLATE NO.	GROWTH-RATE (mm/day)	OBSERVATIONS ON GROWTH AND MORPHOLOGY IN TUBES AND FLASKS
LEPISTA NUDA	1	1.47	Fine mycelial development. Poor growth. Dark colouration of compost following growth suggests poor utilisation. No stranding. Development in tubes of a few dense fluffy white patches in compost and at compost-casing interface.
	247.69	3.42	Mycelial density medium. Strong strand development in casing, tendency to formation, in flasks, of densely packed fine white particles at compost casing interface.
	5	3.84	Dense uniform mycelial growth. Some strand development in casing. Good utilisation of compost.
	6	1.93	Slow growth. Development of some strands in casing and compost.
	7	3.8	Dense uniform growth. No strand formation.
	8	3.62	Medium density mycelial growth. Marked tendency towards strand development in compost.
	9	3.76	Strong, though fine, mycelial growth in compost and casing, strong lilaceous colouration particularly at the casing surface in flasks. Good utilisation of compost (assessed by colour following growth). Stranding in flasks, marked tendency to strong development of strands in the compost of all tubes.
	10	3.93	Dense, fine, white hyphal development through compost. Dark colour of colonised compost following incubation, indicitive of poor utilisation. Slight strand development in tubes. Formation of compact balls of hyphae in the compost of the flasks.

SPECIES	ISOLATE NO.	GROWTH RATE (mm/day)	OBSERVATIONS ON GROWTH AND MORPHOLOGY IN TUBES AND FLASKS
LEPISTA NUDA	11	1.92	Strong growth in compost and casing, this is carried almost to the extent of tissueing in the flasks, with a bright purple colouration of hyphae especially at the casing surface. Development of strands in the casing of both tubes and flasks. Strong development of strands in the compost of one tube. Dark brown/black stain from tissued hyphae pressed against the flask-wall at one point, is a possible indication of strong tyrosinase activity.
LEPISTA SAEVA	1	2.52	Dense, though slow, mycelial growth. Good colonisation, though dark colour of compost following several months incubation suggests poor utilisation. Some strand development in compost of tubes. In flasks numerous small, compact balls of hyphae developed (These spheres had brown centres indicating tyrosinase activity).
	248.69	2.36	Dense slow growth, through the compost, resulting in colonisation almost to the extent of tissueing in flasks. Cultures in tubes showed strong development of strands in the casing, but to a greater extent in the compost. Growth through the casing was generally weak.
	5	3.16	Dense white mycelial growth. Compact tissueing along straws and occasionally as balls of hyphae in flasks, with formation of dense white particles scattered in the upper region of the compost in tubes.
	6A	1.57	Medium dense though very slow mycelial growth in tubes, somewhat stronger development in flasks with unusually strong growth through the casing layer. No strand development.

SPECIES	ISOLATE NO.	GROWTH RATE (mm/day)	OBSERVATIONS ON GROWTH AND MORPHOLOGY IN TUBES AND FLASKS
LEPISTA SAEVA	6B	2.97	Medium dense mycelial growth. Some thick strand formation in tubes. Light brown appearance of compost indicates good utilisation. All flasks and tubes contain numberous white compact balls of hyphae, 1 mm dia., in tubes, larger in flasks c.2 x those formed by isolate 1 in flasks. Small dense regions also apparent in tubes (possibly sectors)
CALOCYBE GAMBOSA	1 2		Tubes inoculated with both strains 1 & 2 did not support growth.

Observations were made on the growth-rate of single L. nuda and L. saeva isolates (No. 1) on a range of formulated 'short-composted' substrates (Table 13.1), relative to rate of growth achieved on a commercial mushroom growers compost. The substrates were subdivided, one-half underwent a secondary thermophilic fermentation (peak-heat) and pasturisation. Analysis for total Kjeldahl nitrogen and moisture was made.

TABLE 13.1 Growth on Short Composted Substrates.

SUBSTRATE †		ANALYSIS		Lepista nuda 1	Lepista saeva 1
Non-Peak Heated (N-PH) Peak Heated (PH) (Pasteurised)		g % N (Kjeldahl Nitrogen)	Moisture %	per cent of control	per cent of control
Commercial mushroom compost (control)	PH	2.3	65	100	100
Short composted Barley Straw and Chicken manure	N-PH	2.44	79	52	67
	PH	2.32	77	Tr	71*
Short composted Barley straw, chicken manure and wood shavings	N-PH	1.92	76	89*	119*
	PH	2.02	75	82	79*
Spent compost, wood shavings, and compost activator.	N-PH	1.76	67	82*	83
	PH	1.72	66	54	48
Spent compost, wood shavings, malt dust compost activator.	N-PH	1.53	63		
	PH	1.67	71	63	75
Wood shavings, chicken manure, compost activator	N-PH	1.68	70		
	PH	1.57	78	75	52
Wood shavings, spent compost, chicken manure and compost activator.	N-PH	2.13	66	75*	63*
	PH	1.74	65	54	52
Wood shavings grass cuttings.	N-PH	0.7	76	68	71*
	PH	1.73	71	75	74
Wood shavings and compost activator	PH	0.99	68	82*	103*
Spent compost (obtained from beds of the cultivated mushroom)		1.94		Ingredients forming the larger proportion of each substrate are listed first.	
Malt Dust		0.462	Dry		

TABLE 13.1 cont:

SUBSTRATES Non-Peak Heated Peak Heated (Pasteurised)	ANALYSIS g % N (Kjeldahl Nitrogen)	ANALYSIS Moisture %	
Wood shavings. (Pine or spruce shavings formally used as stable litter and partly rotted)	1.1	73.1	Ingredients forming the larger proportion of each substrate are listed first.
Chicken manure (Broiler chicken litter containing wood shavings)	3.35	66.2	

† Duplicate tubes for each substrate were autoclaved (see materials and methods) and inoculated individually with a few grains of the appropiate grain spawn.

* Dense hyphal growth of isolates. The Lepista saeva strain on these substrates produced strong strand development leading to compact balls of hyphae in the surface compost adjacent to the inoculum (possible primordia).

SECTION 7

14:0 FRUITBODY DEVELOPMENT ON A NATURAL AGAR MEDIUM

C. gambosa isolates 2, 3 and 5 and L. saeva isolates C.B.S. 248.69, 5 and 6B produced only vegetative growth over the agar surface. C. gambosa isolates 3 and 5 during incubation produced large numbers of orange stellate crystals on the colony surface and finally autolysis took place. L. saeva strain 6B produced well defined radiating strands from the central inoculum to peripheral sites of growth, but did not show further development.

L. nuda isolate 13 strongly colonised the agar following twenty-one days incubation forming a dense development of purple aerial hyphae, but showed no differentation. L. nuda isolate 8 produced strong hyphal growth over the agar medium. At five months following inoculation the colony surface developed dense aggregations of hyphae, possible fruitbody initials, however, these did not show structural differentation nor did they develop further.

L. nuda isolate 9 formed primordia and small immature fruitbodies in the third month following inoculation initially at the mycelial-agar interface along the sides of the flask (Plates 11–19). The sporophores, the largest of which attained a size of 10-15 mm, produced a reddish-brown pileus and speckled lilaceous stipe, supporting a number of thick, soft, well spaced lamellae. Microscopic examination revealed no discernable hymenium and basidia - no spore print was produced. Development was gymnocarpic, the immature fruitbodies differentiated from the terminal portion of stalk-like growths, with the larger specimens the base of the stipe expanding downwards to encompass the supporting structure.

Large numbers of branched stalk structures which had the appearance of antler-like growths developed in the area around the sides of the flask where the agar had shrunk back from the flask walls. Small immature sporophores were generally produced from the terminal portions of these branches. Dense masses of unbranched stalk structures were produced in areas of the main colony on the upper agar surface during the fourth month, however, these structures did not differentiate further.

LEPISTA NUDA FRUIT-BODY PRIMORDIA FORMED ON MALT/YEAST EXTRACT AGAR.

PLATE 11.

Development of twin primordia between the agar and flask-wall.

SCALE 1 cm.

PLATE 12.

Primordial development on the agar 'wall' within the gap created by contraction of the agar.

SCALE 1 cm.

PLATE 13.

Fruit-body primordia 'pinched off' from the distal part of stalk-like growths formed from the surface hyphae.

SCALE 1 cm.


50

LEPISTA NUDA FRUIT-BODY PRIMORDIA FORMED ON MALT/YEAST EXTRACT AGAR.

PLATE 14.

Indication of the large number of primordia which formed in each flask.

SCALE 1 cm.

PLATE 15.

Well developed primordia from several flasks.

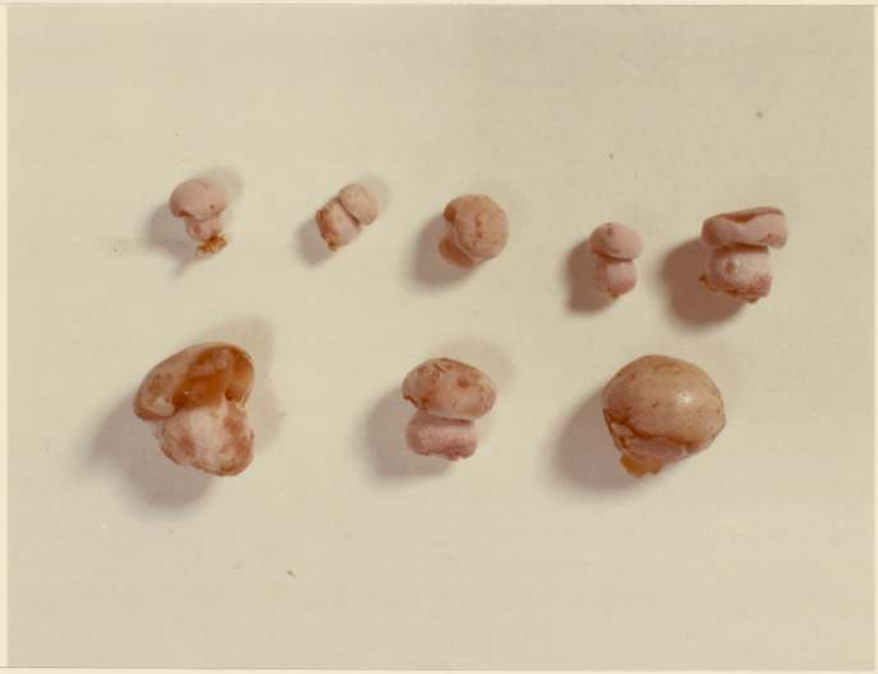
SCALE 1 cm.

PLATE 16.

Clump of 3 primordia. Lamellae at early stage of development.

SCALE 1 cm.


150
APPROX.
VOLUMES
100
50



LEPISTA NUDA FRUIT-BODY PRIMORDIA FORMED ON MALT/YEAST EXTRACT AGAR.

PLATE 17.

Primordia developing from the terminal region of branched 'antler-like' growths. (Arrowed).

SCALE 1 cm.

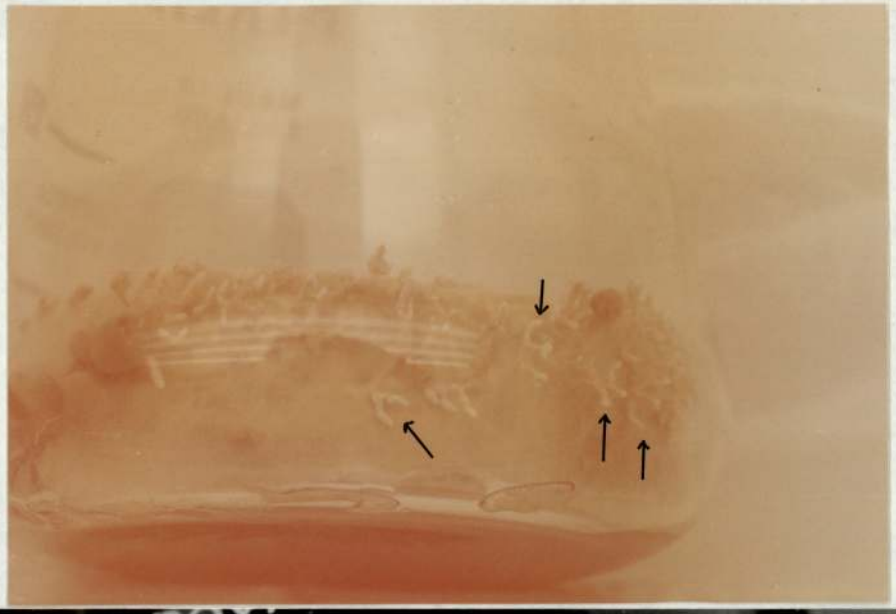
PLATE 18.

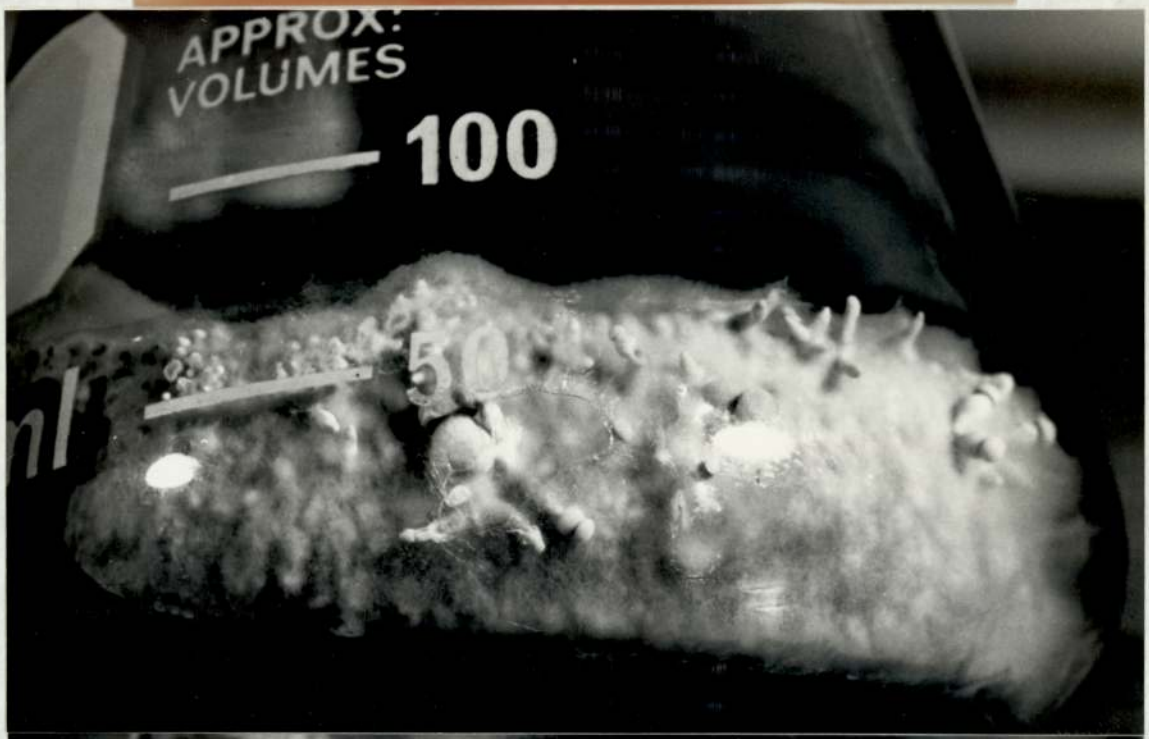
and

PLATE 19.

Differentiation of numerous primordia.

SCALE 1 cm.




APPROX.
VOLUMES
100
50

In several of the flasks submerged masses of externally purple pigmented tissue, c.1 cm diameter, developed within the volume of agar, causing splitting of the agar, however, the formation of these structures in the 5th month occurred too late for any direct correlation with the main phase of sporophore development.

14.1 Fruitbody Formation on a Solid Substrate

All three L. saeva isolates colonised the compost and casing layers, particularly strong growth through the compost was made by 1 and 6B, these isolates also made best progress with growth through the casing materials together with some development of strands within both compost and casing. Growth into the casing layers was considerably less vigorous than that achieved by L. nuda, in this respect the peat-chalk casing mix and loam soil proved to be the most suitable materials.

Colonisation by the L. nuda isolates within the compost progessively intensified, mycelial density was not initially as marked as that achieved by L. saeva strains. Growth from the colonised compost into the leaf preparations, peat-chalk and soil casing layers was generally vigorous and persistent. Over a period of several weeks following colonisation of the two successive layers of casing materials, in each case the L. nuda isolates formed a dense surface layer of purple tissued hyphae. This hyphal development was possibly analogous to the 'dense, flakey and crusty' growth referred to by Constantin and Matruchot .

L. nuda isolates 8 and 9 produced fruitbodies in each of the four treatment containers (Plates 20-28), isolate 6 produced only a single fruitbody.

Dissection of a container of isolate 8 shortly following the advent of fruitbody production revealed 15 fruitbodies at various stages of maturity. The fruitbodies generally arose within the 1st casing layer, frequently at the compost interface, and also at the sides of the container where contraction in volume of casing and

P L A T E 20.

Lepista nuda sporophore produced in culture. (Isolate No.8).

Note: Strong development of surface purple hyphae.

SCALE 3 cm.

P L A T E 21.

Sporophores of Lepista nuda, (Isolate No.9), formed in culture. Abnormally developing fruit-bodies produced from a central mass of tissue.

SCALE 1 cm.

P L A T E 22.

Sporophores of Lepista nuda. (Isolate No.9 left; Isolate No.8 right from Plate 9 at an earlier stage).

SCALE 4 cm.

PLATES 23 & 24.

Lepista nuda Isolate 8.

23. (Left) Sporophore emerging from side of container.

SCALE 1 cm.

24. (Right). Sporophore (from 12 above) removed from container, showing large mass of basal tissue.

SCALE 1 cm.

PLATES 25 & 26.

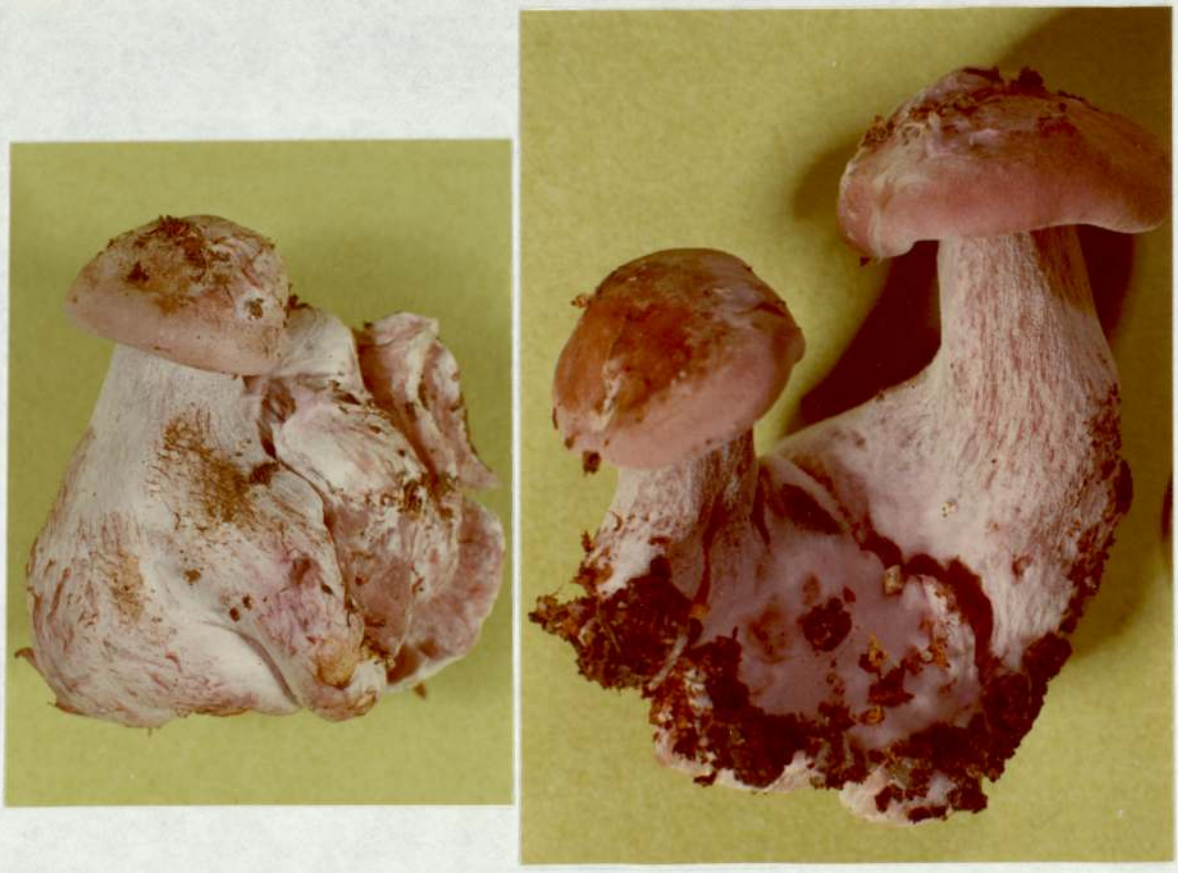
Lepista nuda Isolate 8 .

25. (Left). Immature sporophore associated with large mass of basal tissue.

SCALE 2 cm.

26. (Right) Immature sporophores with strongly developed colouration.

SCALE 2 cm.



P L A T E 27.

3 Fruit-bodies of Lepista nuda (Isolate 9) with relatively long stipes and small caps. Note: 'Foot' comprising basal tissue mass.

SCALE 2 cm.

P L A T E 28.

Malformed sporophore of Lepista nuda (Isolate 9), associated with a mass of immature sporophores. These further developed to give those of Plate 10.

SCALE 1 cm.

compost had caused a gap to appear. Fruitbodies arising centrally required to breakthrough the surface 'skin' of hyphal tissue; this surface layer greatly restricted water uptake by the casing, applied over the casing surface, and limited free gaseous diffusion, its presence was probably of importance in producing conditions within the casing conducive to fruitbody formation.

All immature fruitbodies were associated with a bulb of tissue (Plates 25 & 26), in one case five immature fruitbodies arose from a single common bulb of tissue, generally the stipe increased in width towards the base forming a continuation with this basal bulb. The tissue mass became integrated into the base of the stipe as the fruitbody enlarged and matured, giving rise to the 'blue-foot' which appeared as an extension of the stipe generally at an obtuse angle to the main body of the stipe (Plate 24).

Strong development of strands occurred within the casing layer during fruitbody formation. Those strands associated with the base of the developing sporophores were particularly large and probably constituted the rhizomorphs referred to by Constantin and Matruchot.

The small fruitbodies left to mature enlarged very considerably in size from 1-1½ inches to a height of c.5 inches, sizes equivalent to those found in nature. Allowed to expand the fruitbodies produced the typical recurved cap and 'tricholomatoid' appearance attaining eventually unnaturally large proportions, a phenonmenon recorded as gigantism by Constantin and Matruchot.

A number of fruitbodies in one container of isolate 9 were formed atypically (Plate 21). These arose from a central mass of tissue. A number of the more mature fruitbodies developed a stipe curvature sufficient to invert the cap. In those specimens where the cap made contact with the basal tissue, fusion occurred at the point of contact, where contact was made with the hyphae on the casing surface, strong development of several thick strands occurred at that point.

Fruitbodies allowed to develop produced a mature hymenium from which a number of spore prints were made.

Grain Spawns

Generally good colonisation of grain spawn media was achieved by L. nuda and L. saeva isolates.

The suitability of the grain spawn media was roughly related to its physical properties. Rye proved superior in this respect due to the hardness of the grain preventing splitting during cooking procedures, and size, in terms of convenience in washing and dispersal as an inoculum. Wheat grain was prone to splitting, exudation of starch causing clumping of grain, sticking to the base of the flask and considerably increased chances of contamination. Millet was less manageable in washing and soaking procedures, the inoculum consisted of small compact aggregations of millet grain and mycelium. The milo grain included a large proportion of split grains which were difficult to eliminate and causedfree starch formation, milo grain proved unsatisfactory for this reason.

SECTION 8

Effect of Oxygen and Carbon dioxide tension on hyphal growth: Experiments with the gas diffusion column

15:0 INFLUENCE OF OXYGEN TENSION

The direct effect of oxygen on the rate of mycelial growth of two isolates each of L. nuda (7 & C.B.S. 247.69) and L. saeva (5 & C.B.S. 248.69) was studied for gas mixtures grading in composition from atmospheric (21.0% oxygen) to 0.3% oxygen (Figures 15.1 & 15.2). The lower level represented the maximum level of this gas as an impurity in the supplies of commercial nitrogen. Nitrogen gas was run against air, producing a countercurrent flow, in which the nitrogen acted as an inert dilutent replacing the oxygen as its concentration fell along the length of the column.

In general maximal growth was attained at very low concentrations of oxygen. The oxygen concentration at which growth

FIGURE 15.1
EFFECT OF OXYGEN CONCENTRATION ON GROWTH RATE

0-21 PER CENT OXYGEN

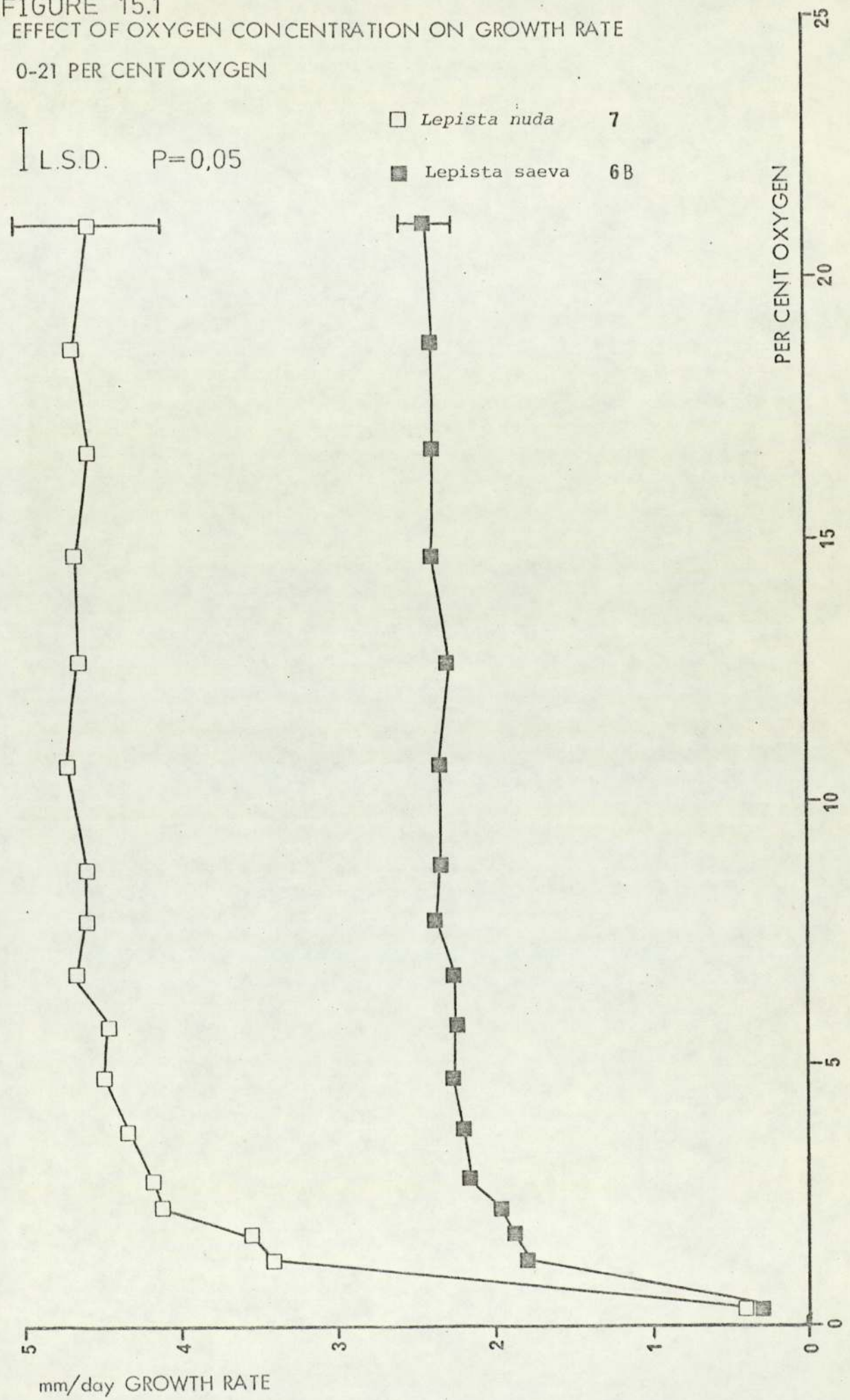
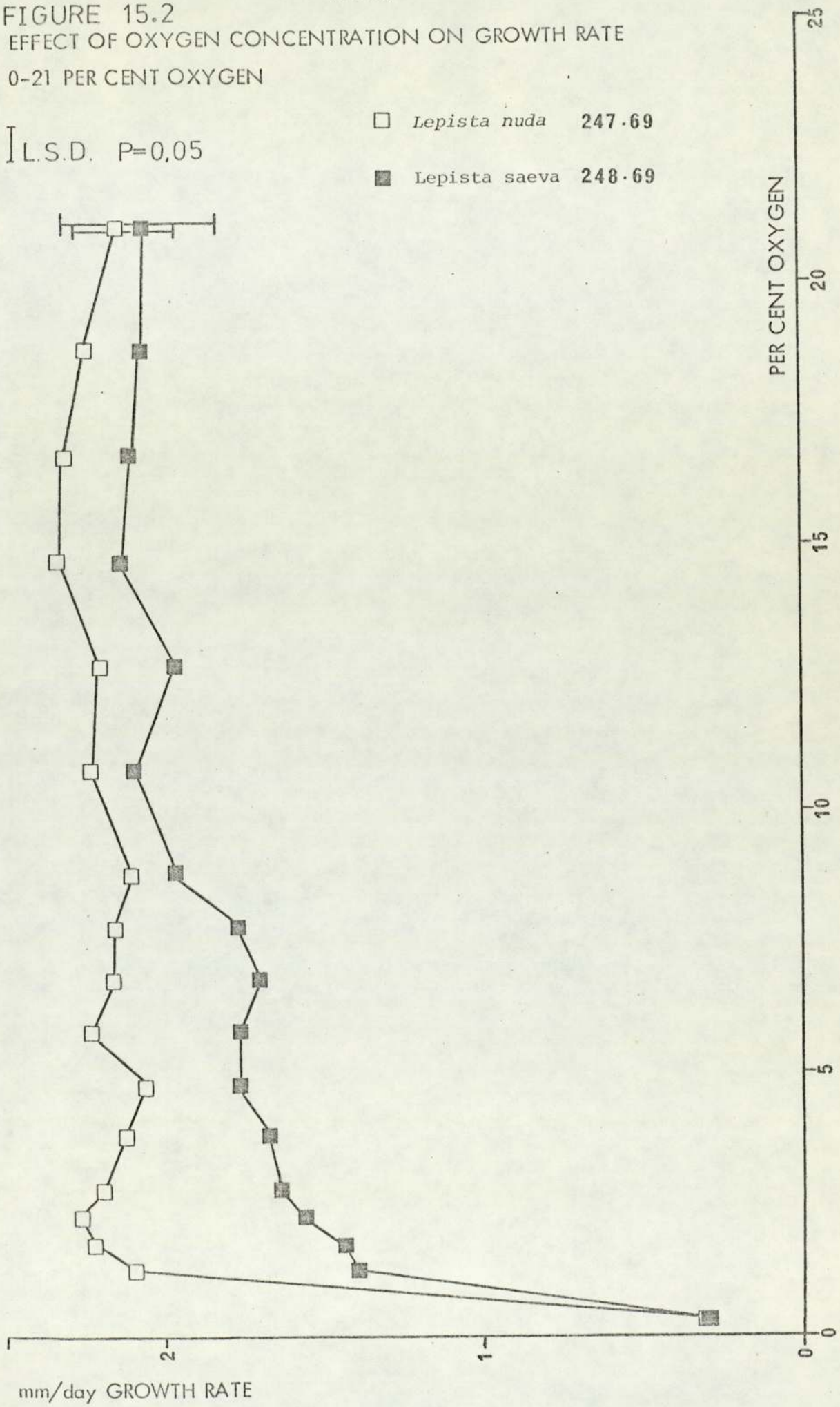


FIGURE 15.2
EFFECT OF OXYGEN CONCENTRATION ON GROWTH RATE
0-21 PER CENT OXYGEN



was reduced to half maximal is considered to be important in determining those oxygen concentrations which would act as an effective constraint to growth in nature. Low oxygen tension could occur in water films, in water-logged soils and at the core of soil crumbs or larger soil aggregates.

Maximal growth of L. nuda isolate 7 was maintained until an oxygen concentration of 2.8%, at this value growth was significantly ($P = 0.05$) reduced to that achieved at atmospheric levels (21.0%). A reduction to half maximum growth rate was produced at c. 0.8% oxygen. Only slight aerial hyphal growth was produced at 0.3% oxygen.

Growth rate of the isolate C.B.S. 247.69 was approximately half that of isolate 7 on the glucose-asparagine agar medium. Near maximal growth was achieved with a reduced oxygen tension of 1.25%, growth at this value was just significantly ($P = 0.05$) below that at atmospheric levels. Half maximal growth was produced at similar values, c. 0.7% to that causing 50% growth inhibition in isolate 7.

L. saeva stram 5 demonstrated a significantly inhibited growth rate ($P = 0.05$) at 4% oxygen. A reduction to half the rate of colony extension occurred at c. 0.9% oxygen. No growth took place at 0.3% oxygen. Isolate C.B.S. 248.69 showed a significant ($P = 0.05$) reduction in rate of hyphal extension at approx., 8.0% oxygen in comparison with that at atmospheric levels. A gradual and progressive reduction in growth-rate from maximal values may indicate instability of this isolate under oxygen stress, however, the oxygen concentration required to retard growth to half maximum was very low at approximately 1%, a similar value to that obtained for isolate 5. Some aerial hyphal development off the inoculum plug occurred at 0.3% oxygen.

In conclusion growth of both species was not significantly inhibited until oxygen concentration was reduced to 4%. That concentration causing a 50% reduction in growth may be considered to be a more reliable measure of the tolerance of the fungus to variation in oxygen concentration. This value for all isolates was in the region 0.7 - 1.0% oxygen. Slight growth was apparent for some isolates at 0.3% oxygen.

15:1 Influence of Carbon Dioxide Tension

Growth response to higher than atmospheric levels of carbon dioxide (0.03%) was considerably more variable than that produced by reduced oxygen tension.

L. nuda C.B.S. 247.69 and L. saeva C.B.S. 248.69 were cultured in atmospheres containing carbon dioxide in the range 0.69 to 12.0% (Figure 15:3). Carbon dioxide replaced nitrogen in the gas mixture. Significant (P = 0.05) inhibition of the L. nuda isolate was evident in the approximate concentration zone 3.2 to 6.12% CO_2. Growth inhibition between 6.0 to 10.5% CO_2 showed a linear reduction directly proportional to increase in carbon dioxide concentration. Fifty per cent inhibition of growth was produced at a carbon dioxide tension of 9.5%.

A significant (P = 0.05) reduction in growth of the L. saeva isolate occurred between 4.5 to 5.0% CO_2 with marked growth inhibition beyond 9%. Half maximal rate of growth was produced with a carbon dioxide level of 10.6%.

Rates of hyphal extension for L. nuda isolate 7 and L. saeva isolate 6B were determined over a range of carbon dioxide tensions between zero and five per cent (Figure 15.4).

Growth inhibition of L. nuda 7 was marked above *c.* 2.0%, maximum growth was recorded at 0.35% CO_2, however, this was not significant with respect to zero per cent. Considerable growth inhibition was apparent over the range 0.35 to 3.0% CO_2, at values above 3% growth rate was not strongly affected. At 0.6% CO_2 growth was significantly (P = 0.05) inhibited in relation to the zero per cent value. Fifty per cent inhibition of growth was produced at 2.4% CO_2.

Growth reduction of the L. saeva isolate 6B was more directly proportional to carbon dioxide concentration. Growth was significantly (P = 0.05) reduced from maximal at 0.7%, however, a 50% reduction in growth rate was only achieved at values in slight excess of 5% CO_2.

FIGURE 15.3

EFFECT OF CARBON DIOXIDE CONCENTRATION ON GROWTH RATE
0-12 PER CENT CARBON DIOXIDE

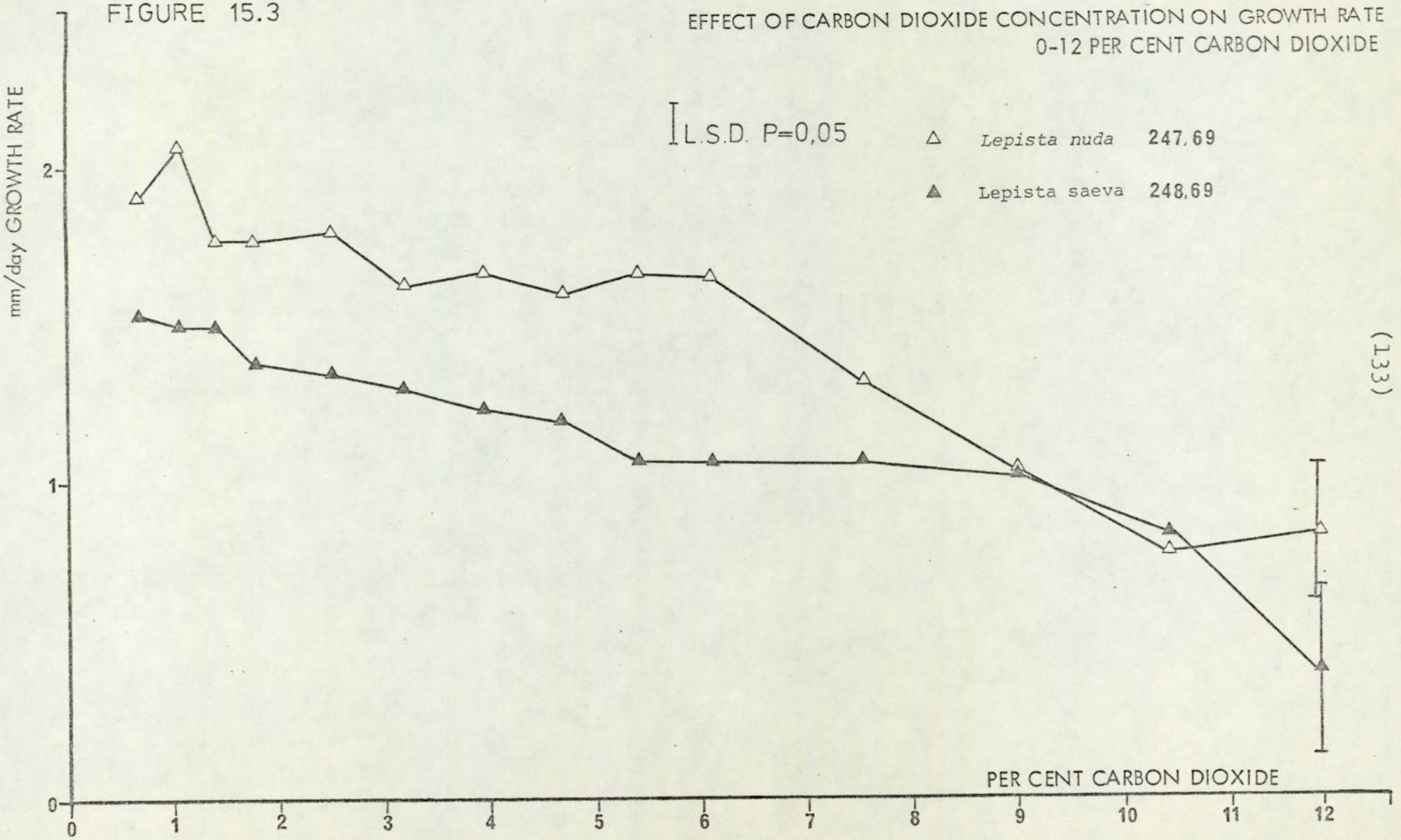


FIGURE 15.4

EFFECT OF CARBON DIOXIDE CONCENTRATION ON GROWTH RATE
0-5 PER CENT CARBON DIOXIDE

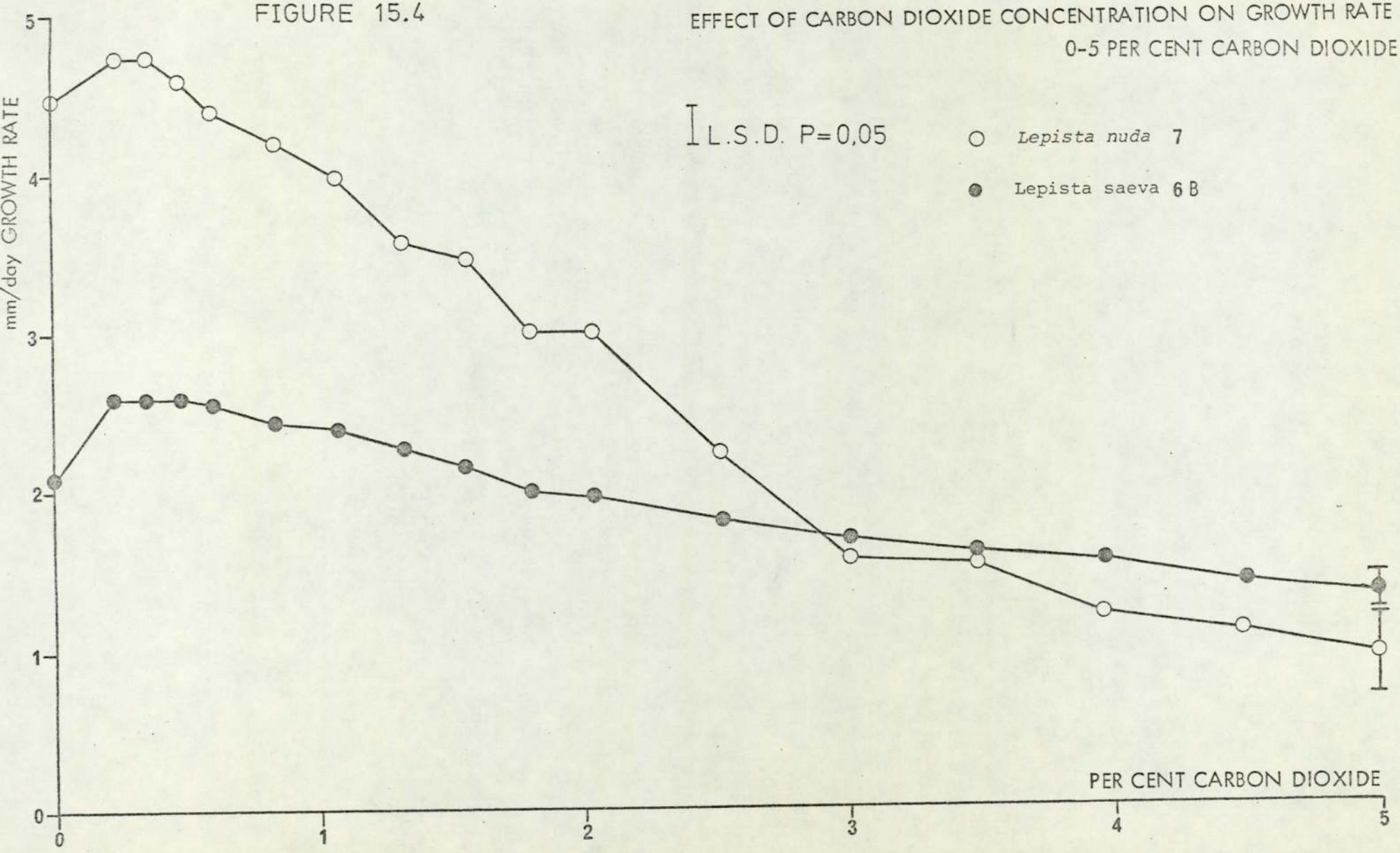
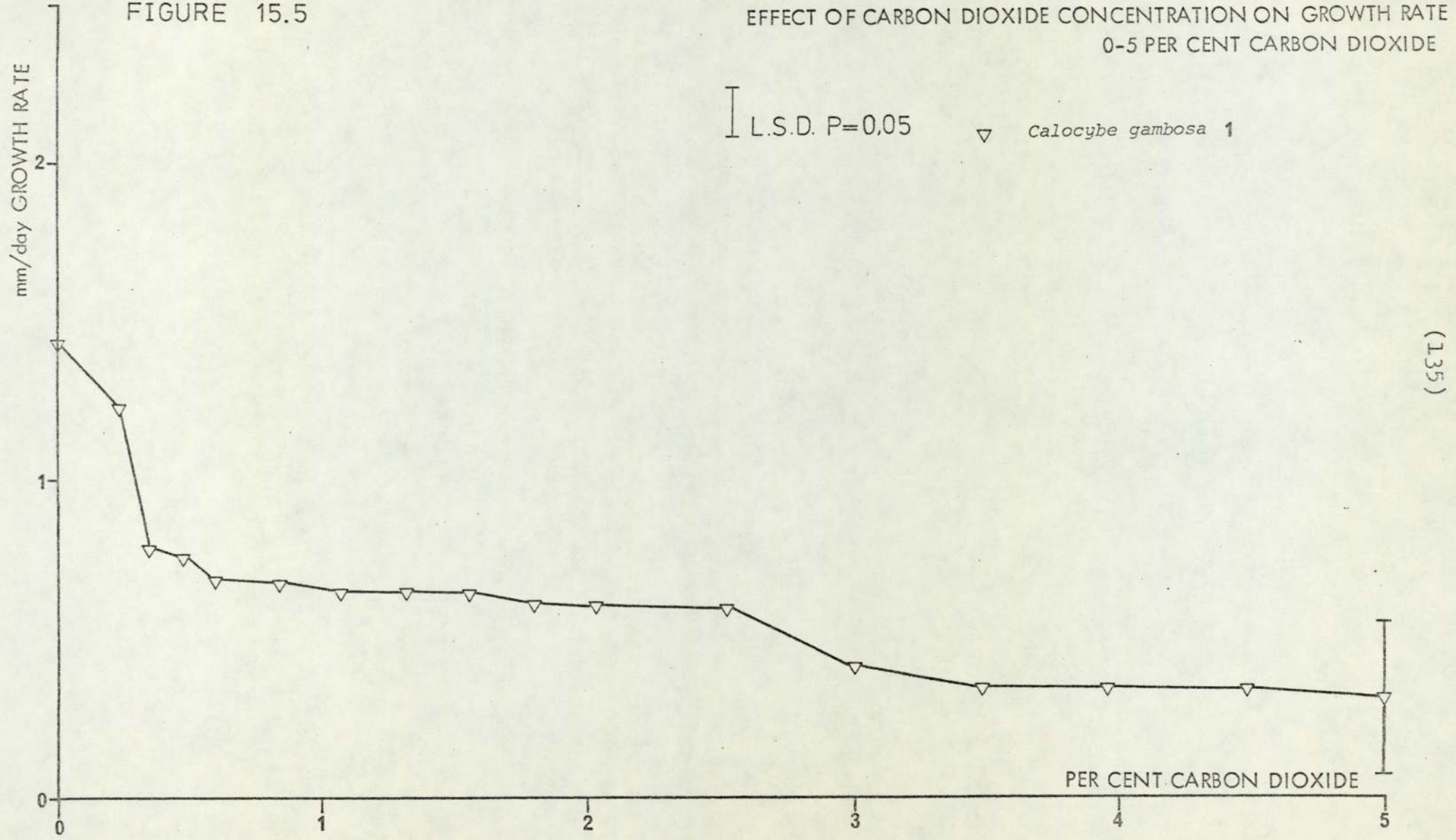


FIGURE 15.5

EFFECT OF CARBON DIOXIDE CONCENTRATION ON GROWTH RATE
0-5 PER CENT CARBON DIOXIDE



Growth response for a single isolate of Calocybe gambosa (I) was ascertained at carbon dioxide concentrations with the range 0-5% (Figure 15.5). Growth of this strain was strongly inhibited at 0.6% CO_2, growth rate was significantly inhibited (P=0.05) at 0.25% CO_2. A reduction to half maximum growth rate occurred at 0.55% CO_2.

In conclusion the inhibiting effect of carbon dioxide was progressive during incubation, growth inhibition rated over long incubation periods was cumulative as represented by the growth rates given here; inhibition at low carbon dioxide tensions was only clearly apparent following 25 days incubation of the L. nuda and L. saeva isolates at 0-5% CO_2.

In assessing the effect of carbon dioxide on growth of cultures, factors which also require consideration in addition to colony age, include nutrient status of the cultures, hyphal density and growth rate. The faster growing and more dispersed colonies of L. nuda 7 were more susceptible to inhibition by carbon dioxide than the slower developing and denser cultures of L. nuda C.B.S. 247.69 and L. saeva C.B.S. 248.69.

DISCUSSION

AND

CONCLUSIONS

DISCUSSION

The present study was undertaken to assess the physiological requirements of the three species L. nuda, L. saeva and C. gambosa for vegetative growth as a prerequisite to an assessment of the additional nutritional and environmental requirements forthe formation of fruitbodies.

The high mycelial dry-weights obtained for L. nuda and L. saeva are in sharp contrast to the very low yields given for growth of these species on synthetic media by a number of previous investigators (Kőgl and Fries 1937, Modess 1941, Norkrans 1950, 1953, Lundeberg 1970). Explanations for yields below 0-15mg frequently obtained are difficult to provide. It is considered that although the isolates obtained by these authors were generally from coniferous locations and may have been less adaptable to growth in synthetic media, the isolates showed a general inability to utilise the media components provided. Separate sterilisation and addition of carbon and nitrogen components, preferably with sterilisation achieved by filtration, was stressed during this study; autoclaving sugars and phosphates or sugars and nitrogen sources is known to promote formation of inhibitors (Cochrane 1958), and associated detrimental effects including hydrolysis of carbon compounds or deamination of nitrogen sources and exhaustion of buffer capacity.

Initial experiments showed that L. nuda and L. saeva were strongly inhibited where glucose with asparagine or glucose with ammonium tartrate were autoclaved together in media (yields below 35 mg following 30 days incubation). The studies of Lilly and Barnett (1951) have indicated that autoclaving glucose-asparagine medium at neutral or alkaline pH has resulted in the precipitation of important metallic ions including Fe, Al, Ca, Ba, Mg, Mn and Zn from the medium. Although it is not suggested that in all these studies combination of such factors is the sole explanation, it is of interest to note that Lamb (1974) obtained considerably improved yields to these investigators with an L. nuda isolate from a pine plantation in a simple synthetic medium where these precautions in sterilising media components were strictly adhered too.

In general the denser hyphal growth shown by L. saeva isolates was reflected in higher dry-weights than those achieved with L. nuda strains on the most favourable sources of carbon and nitrogen. Highest yields of L. saeva isolate 6B were up to one-third in excess of those obtained for L. nuda isolate 7 on the best carbon and nitrogen sources. This is clearly shown from the yields obtained in the C:N ratio studies on the standard glucose-asparagine medium (Figures 6.3, 6.4, 6.5 & 6.6). The yields obtained in C:N ratio studies and analysis for residual sugar following growth of L. saeva 6B on a range of amino-nitrogen sources (Table 8.1) indicate that this species was able to produce good growth until the majority of carbon and nitrogen in the medium was utilised, in this respect efficiency of growth exceeded that of the corresponding L. nuda strains.

The Economic Coefficient is determined from the formula:

$$\frac{\text{Mycelial dry weight (g)}}{\text{Carbohydrate consumed (g)}}$$

Values derived for L. nuda isolate 7 from carbon: nitrogen ratio studies indicate an E.C. of 0.4 - 0.5, these figures approximately agree with those obtained for Lambs (1974) isolate of 0.45 - 0.55 on the best carbon sources. Both sets of figures assume complete utilisation of the carbon source. The E.C. value for the L. saeva isolate (6B) derived from yields on amino-acid nitrogen sources where analysis for residual glucose was made is 0.73, this value agrees well with a figure of 0.71 obtained from C:N ratio studies, where complete utilisation of sugar is assumed. High Economic Coefficients are generally a feature of Basidiomycetes (Burnett 1968).

Carbon Sources

Comparison of growth between L. nuda and L. saeva isolates over a wide range of carbon and nitrogen sources suggested a higher level of nutritional adaptability by L. nuda. L. nuda isolate 7 produced better growth than the corresponding L. saeva isolate 6B on the carbon sources pectin, galactose and the polyols dulcitol, mannitol and sorbitol (Figures 7.1 & 7.2). Highest dry-weights were produced on the hexitols derived from the sugars supporting

heaviest yields (i.e. mannose-mannitol, glucose-sorbitol), L. saeva produced markedly improved growth on ribose in comparison with L. nuda. Both species appear to possess a wide-range of enzymes enabling utilisation of disaccharides including sucrase (invertase) maltase, cellobiase, trehalase and melibiase. Raffinose is split either to fructose and melibiose by sucrase or to galactose and sucrose by attack at the alternative linkage. The high yields obtained on raffinose in comparison with poor utilisation of galactose and melibiose by these species, when present as sole carbon sources, indicates that provision of an alternative carbon source by attack on raffinose provides energy for induced enzyme systems required for metabolism of galactose and melibiose.

It is probable that these species can synthesise the sugars mannitol, trehalose and glycogen which have been detected in significant quantities in the mycelium and fruitbodies of Agaricus bisporus (Hughes 1961, Holtz 1971, Hammond and Nichols 1976).

Both species produced good growth on starch indicating strong production of α & β amylases. Dense colony mats were produced in the combined starch + maltose + glucose medium, combination of pectin, galactose and glucose improved growth for L. nuda but not for L. saeva. Combination of ribose, arabinose and xylose did not improve their utilisation by either species, growth on mannose, sorbose and arabinose approximately equalled the expected yield on the mannose fraction.

Poor utilisation of the organic acids, citrate, malate tartrate, succinate and oxalate is general within the fungi, the exception being good growth obtained by Agaricus bisporus on these Krebs cycle acids (Bohus 1959). Large amounts of oxalic acid may be accumulated by this fungus (Tsao 1963). Inability to utilise these substances is probably due to their inaccessibility caused by formation of insoluble Na or Ca salts, or impermeability due to ionisation of the cell-wall, cell membrane or cell colloids in the normal physiological pH range. Improved utilisation may occur at low pH values (Cochrane 1958).

Competitive inhibition between the sugars glucose and fructose and mannose, glucose and xylose has been recorded for T. nudum by Reusser, Gorin and Spencer (1960) and Cirillo and Grayson (Cirillo 1961). The sugars are preferentially utilised in the order stated.

The C. gambosa isolate generally produced best yields on the hexose sugars and disaccharides present (Figure 7.3). Xylose proved a favourable carbon source and good growth was produced on the combination ribose, arabinose and xylose. Yields given represent actual mycelial dry-weights produced and do not include weight of the inoculum mycelial-agar-plug which has been frequently included in yield tables in the literature, this may give a mistaken impression of the extent of growth when the actual yields recorded are low.

Cellulolytic Activity

All species showed moderate yields where cellulose was present as sole carbon source. Cellulolytic ability was assessed for a number of strains within each species as the capacity of the fungus to lyse finely divided cellulose producing zones of clearing in a milk-white opaque medium. The results (Table 11.1) indicated considerable variation within the respective groups (L. nuda range 17 to 49%; L. saeva range 28 to 100%; as unit values on 0.25% cellulose agar). Clearing could only be assessed for the C. gambosa isolates on 0.25% cellulose addition.

This method provides a convenient means of assessing cellulolytic capacity for a large number of fungi with adequate replication of individual treatments. Ability to breakdown 72 hr ball-milled cellulose which is present in a finely divided state cannot be directly correlated with attack on native cellulose. Organic nitrogen sources such as asparagine and yeast extract included in the medium may reduce the extent of cellulase production (Malik 1970). The extent of clearing will also be directly related to the density of the mycelial mat developing above the agar, in this

respect results may be bias in favour of greater cellulase production by L. saeva. Growth and cellulose clearing in Petri-plates, however, indicates that L. saeva produces overall higher levels of cellulase. Initial experiments in which L. nuda and L. saeva isolate 1 were grown on beech and spruce sawdust agars with synthetic salts addition indicated rapid surface hyphal growth and slow clearing of the agar.

The inability of certain investigators (Reusser, Spencer and Sallans 1958 b, Lundeberg 1970) to demonstrate cellulolytic ability by L. nuda isolates may be due to only slight mycelial development by their strains under the conditions of culture. Norkrans (1950) and Lindeberg (1946) have been able to demonstrate cellulose breakdown by all three species following incubation on sterilised Glyceria straw.

Polyphenol Oxidase Enzyme Levels

Assessment of polyphenol oxidase activity as a measure of an individual species capacity to degrade lignin also indicated variability between strains (Table 12.1), this is most apparent in considering the inhibitory effect on growth of gallic and tannic acid or their breakdown products. Discolouration of gallic and tannic acid media in the Bavendamm test is considered to be due to the production of ortho-and para-quinones or quinone-protein complexes (Lindeberg and Fåhraeous 1952, Lindeberg and Holm 1952). The formation of intermediate orange coloured complexes observed for C. gambosa and single isolates of L. nuda and L. saeva has been observed for Agaricus bisporus (Stoller, 1954) a strong producer of polyphenol oxidases.

Production of a coloured zone on gallic and tannic acid media is considered to indicate p-diphenol oxidase or laccase activity (Walker 1975, Lindeberg 1948). The properties of tyrosinase and laccase enzyme fractions which constitute the polyphenol oxidase component of Basidiomycetes can be summarised as:

(a) Tyrosinase, oxidises monophenols (i.e. Tyrosine, p-cresol) and ortho-diphenols (i.e. catechol, pyrogallol). Oxidation of tyrosine leads to the formation of black melanin pigments.

(b) Laccase, oxidises ortho-diphenols and para-diphenols (i.e. para-phenylamine diamine, guiacol). This enzyme is sensitive to inactivation by carbon monoxide.

The level of individual enzyme systems is generally assessed by colour reactions on mono-phenols (tyrosine, p-cresol) indicating only tyrosinase activity and on para-diphenols (p-phenylaminediamine, guiacol) indicating only laccase activity and occassionally by the use of carbon monoxide selective for the presence of laccase. Discolouration on gallic and tannic acid and on catechol does not clearly differentiate between the contribution made by each enzyme system, however, tyrosinase is considered to be intracellular and laccase extracellular, the Bavendamm test has been demonstrated to show the presence of extracellular laccase (Lindeberg 1948).

It has been demonstrated for Agaricus bisporus that most of the tyrosinase is present in the fruitbodies, the laccase is in the mycelium within the compost, both enzymes have been detected within the strands of the casing layer. (Lindeberg 1950, Turner 1968, 1974). The presence of a tyrosinase system in L. nuda (isolate 7) and L. saeva (isolate 6B) was indicated by the development of black pigments where tyrosine was employed as sole nitrogen source. Black discolouration of submerged mycelium of C. gambosa was also observed. All three produced a strong black discolouration when catechol was added to the culture filtrates. It is suggested that C. gambosa possesses a very strong laccase system but weak tyrosinase production. These enzyme levels appear to be more equally balanced in L. nuda and L. saeva.

Matsubara and Iwaski (1972) have indicated fairly high levels of tyrosinase and laccase in fruitbodies of Lepista subnuda. The laccase enzyme is probably adaptive in nature (similar to cellulase), studies with A. bisporus on a commercial mushroom growers compost have indicated gradually increased levels of laccase until the point at which fruitbody production and maturation occurs (Turner 1967, 1975).

The studies of Waksman and Nissen (1932), Gerrits, Bels-Koning and Muller (1962) and Gerrits (1967) have shown that the

cultivated mushroom, Agaricus bisporus, obtains the majority of its nitrogen when grown in commercial 'synthetic' and horse-manure composts from degrading the fixed (insoluble) nitrogen-rich-lignin-humus complex (that lignin fraction insoluble in 72% sulphuric acid), which has been built-up by fixation of ammonium nitrogen into amino-acids and peptides by the action of micro-organisms during the composting cycle. Ability to degrade the 'lignin-humin' complex within the upper layers of forest and pastureland soils may provide an important source of organic nitrogen compounds for fungi colonising these layers. These substances would be unavailable to fungi lacking polyphenol oxidase enzyme systems.

'Start Glucose'

The importance of 'start glucose' has been indicated by the researches of Lilly and Barnett (1951), Norkrans (1950) and Lamb (1974). 'Start glucose' consists of a trace glucose addition to the medium which is considered to supply sufficient energy for the activation of 'adaptive' enzyme systems as opposed to the normally operative 'constitutive' enzyme systems. These enzymes may include enzymes which split oligo-or polysaccharides at the cell surface or carry these substances(permeases) across the cell membrane for intracellular lysis. Several such enzyme systems are known to have a requirement for glucose (Burnett 1968).

Lamb (1974) obtained highly significant (P = 0.001) increases in yield following addition of trace glucose on the carbon compounds galactose, fructose, maltose, dextrin, glycogen, starch and inulin for his strain of L. nuda, the isolate was apparently unable to accumulate sufficient energy for growth over the 42 day incubation period on these substrates alone for induction of adaptive enzymes to occur. The inclusion of asparagine in the basal medium used in the present studies may have enabled adaptation to a number of carbon sources, however, very little growth was made in control flasks where asparagine constituted the sole carbon and nitrogen source, it is unlikely therefore at the concentration at which it was present it

constituted a significant energy source.

It is probable that repeated subculture of the present isolates on 2% malt extract agar which contains significant levels of maltose, glucose, sucrose and dextrin ensured that the enzyme systems required for the utilisation of these substrates were active, however, Lamb's cultures were isolated onto 2% malt extract and routinely cultured on Hagem's agar, which contains 5g/L malt extract, his isolate of L. nuda was able to show only minimal growth on maltose, sucrose and dextrin. It is possible that the minimal medium employed by Lamb which was deficient in a range of inorganic ions (Na, Zn, Ca, Mn and Cu) could have restricted utilisation of some of these carbon compounds.

Nitrogen Sources

The results for the growth of the three species on organic, ammonium, nitrate and nitrite nitrogen-sources indicate that final yields must be considered in relation to pH change in the medium (Figures 8.1-8.3). Utilisation of a few organic nitrogen sources resulted in a net release of ammonia into the medium, which was clearly demonstrated with the metabolism of urea. Poorer growth of L. saeva isolates on urea compared with L. nuda was probably a result of less tolerance of this species to alkaline conditions. Release of ammonia may also occur particularly at high nitrogen levels with metabolism of amide and amino-acid nitrogen sources. This is demonstrated by a shift to higher pH values by L. nuda 8 and L. saeva 5 on propionamide and with C. gambosa isolates on asparagine. A shift to alkaline pH occurred with all species at high asparagine concentrations in C:N ratio studies (Figures 6.1 & 6.2). Metabolism of metallic nitrate salts generally resulted in a shift to alkaline conditions (L. saeva 5 & 6B on pot. nitrate and 5 on sodium nitrate), however, this may be countered by organic acid formation in the medium resulting in a stable pH. This may have part attributed to high yield by L. nuda isolates 7 & 8 and L. saeva 5 on nitrates.

Utilisation of ammonium compounds is recognised to result in the formation of acid conditions. This is particularly so

with metabolism of ammonium salts of strong acids, good growth on ammonium bicarbonate by all species probably resulted from the formation of a weak acid (carbonic acid) with consequent slight effect on pH. The use of combined ammonium salts (i.e. ferric ammonium citrate, magnesium ammonium phosphate or ammonium hydrophosphate) is advantageous in preventing a pH fall with utilisation of the ammonium ion (Bohus 1959) or combination of organic acids as carbon source with ammonium salts to counter the downward pH drift (Cochrane 1958, Nicholas 1965).

All three species utilised nitrate, good utilisation showed a tendency to be strain specific, ammonium was metabolised in preference to nitrate (Nicholas 1965). Growth was inhibited by nitrite, Rawald (1963) recorded his isolate of L.nuda as showing some growth on sodium nitrite, however, the growth produced relative to the nitrogen-free control value was sufficiently slight for this finding to be ignored.

Calocybe gambosa isolates produced best development on the compound sources of amino acids, peptone and casein hydrolysate. High yields were produced by isolate 1 on asparagine, this isolate also generally grew well on ammonium and nitrate nitrogen. Ammonium tartrate proved a moderate source of nitrogen to both, however, poor yields resulted on ammonium bicabonate and urea. This may have been due to toxicity caused by the release of free ammonia.

Comparison of yields of single isolates of L.nuda and L.saeva on a range of 19 amino-acids, amide and compound sources of amino-acids (Figures 8.4 & 8.5) indicated a wider nutritional diversity by the former species, similar to the pattern shown on carbon sources. Nine amino-acids enabled the achievement of high yields by L.nuda, five by L.saeva, those proving unsatisfactory for the latter species were valine, leucine, threonine and arginine. The amides asparagine and glutamine were equally good sources of nitrogen for L.nuda, glutamine compared very poorly with excellent yields achieved by L.saeva on asparagine. The compound sources of amino-acid nitrogen, casein hydrolysate and the amino-acid mix were comparable with asparagine for L.nuda, however, the amino-acid mixture was distinctly less favourable for L.saeva in comparison with casein hydrolysate.

The majority of amino-acids are considered to be converted directly into proteins (Cochrane 1958), however, where amino-acids or amides form the sole source of nitrogen those best able to take part in transamination reactions would be expected to produce highest yields. Asparagine best fulfills the transamination requirement. Casein hydrolysate and the amino-acid mix are most readily converted into protein.

Poor utilisation by L. saeva (isolate 6B) on glutamine and the amino-acid combination was probably due to a rapid fall in pH with their utilisation, possibly due to the formation of oxaloglutaric acid or similar Krebs cycle acids. Metabolism of aspartic and glutamic acids by both species resulted in a shift to higher pH values. Development of acid conditions following metabolism of arginine probably also accounted for low yields by the L. saeva isolate. Metabolism of arginine within the ornithine cycle possibly resulted in the formation of fumarate or associated organic acids resulting in the pronounced pH disturbance.

The C. gambosa isolate produced best development on the compound sources of nitrogen, the amino-acid mix and casein hydrolysate (Figure 8.6). Highest yields amongst the amino-acids were formed on valine and phenylalanine. Glutamic acid and glutamine produced slightly improved growth to that attained on the corresponding amino-acid and amide, aspartic acid and asparagine. Glutamate has been attributed as a growth factor for Tricholoma gambosum (Norkrans 1953)

C:N Ratio

The carbon:nitrogen ratio of natural substrates is difficult to assess due to their inherent heterogeneity; an optimum C:N ratio in vitro was determined for L. nuda and L. saeva of c. 21:1 (Figures 6.2-6.6), a higher ratio is indicated for C. gambosa (Figure 6.1). L. saeva demonstrated an ability to grow with low levels of nitrogen in the medium.

The optimum C:N ratio for vegetative growth may be defined as, "that balance of carbon and nitrogen, at a specific concen-

tration, and in relation to other components of the medium, which places no restriction on metabolism and cell synthesis required for maximal rates of hyphal/ growth". Growth is considered as increase in cell size, weight and number, and is for the purposes of this definition measured as increase in dry-weight. This requirement is a direct reflection of the organisms genetic resources and is related to the organisms ability to obtain and maintain certain minimal proportions of cell components (intracellular 'pools'). Extrapolation to the organisms natural enviroment is difficult, where there are many potential sources/ of cell components with varing levels of accessibility and priority in pathways of utilisation within the cell.

In pure culture an optimum C:N ratio will be related directly to the conditions of culture (solid, semi-solid, stationary liquid or submerged),/ physical conditions prevailing within these culture systems (aeration, agitation,/ temperature) and the duration of the incubation period, for each particular organism. Two important considerations arising from the present studies are the requirement for an individual C:N ratio for each combination of carbon and nitrogen sources where these form the sole source of these elements, and the choice of a C:N balance providing a stable optimum physiological pH throughout/ out the duration of the incubation period, a requirement which is related to approximately equal depletion of carbon and nitrogen sources in the medium. The ability of Tricholoma nudum to synthesise protein or fats in submerged culture has been clearly demonstrated to be related to the level of carbon and nitrogen in the medium (Reusser Spencer and Sallans 1958b).

Influence of Temperature

Isolates of L.nuda and L.saeva examined for effect of temperature/ on growth-rate indicated a generally lower temperature optimum (20°) than previously reported. Temperature profiles and optima appear to be more variable for strains of L.nuda, the good growth apparent for both species at low temperatures (10°C) with some growth at 4°C is consistant with activity/ of these species at low temperatures during winter months; fruiting may be stimulated by light frosts, fruitbodies of L.saeva are known to have been formed while snow is on the ground. The strong staling of five L.saeva isolates/

at temperatures of 27-28°C indicates that this species is unlikely to withstand high surface soil temperatures. The single isolate of C. gambosa tested demonstrated a temperature optimum at 25°C, growth was markedly reduced at 10°C and absent at 4°C.

Influence of Hydrogen Ion Concentration

Growth profiles over a range of hydrogen ion concentrations indicated an optimum for L. nuda at neutral pH and for L. saeva at slightly acid values (c. pH 6.0), the former species showed more tolerance of increased acid or alkaline conditions. C. gambosa indicated a preference for slightly acid conditions.

Vitamin Requirement

Assessment of the growth response of these species to a series of deletions from a complete vitamin solution indicated a clear requirement for thiamine. This is general within the saprophytic hymenomycetes (Lindeberg 1946). Pyrimidine has been demonstrated to be the essential component for Tricholoma nudum and T. gambosum (Norkrans 1950). In addition a partial requirement for biotin and possibly also pyridoxine and folic acid is indicated for L. nuda and L. saeva. Growth of C. gambosa isolates in this series appeared to be suppressed possibly by choline chloride and inositol additions to the complete vitamin mix, or the use of a charcoal purified asparagine preparation.

Metal Ion Requirements

The complete synthetic medium proved generally superior to the basal solution and equivalent to the basal solution plus leaf litter or manure ash additions. Supplementation of the basal solution with the synthetic mineral solution or zinc generally suppressed growth, calcium at trace addition slightly removed the inhibition caused by zinc. It is probable that the calcium component of the ash additions and the high level of calcium in the complete medium enabled high yields by antagonism of the effects of high levels of metal ions (suppression of the inhibition produced by zinc is indicated from the data) or metabolic staling substances

accumulating in the media. An absolute requirement for calcium is therefore not indicated by growth on the basal solution from which it was absent, however, addition of calcium is advantageous for reduced growth inhibition of these species under the conditions of high nutrient status and direct proximity to accumulating toxic metabolities associated with stationary liquid culture.

Ecological Considerations

L. nuda isolate 8 was less demanding in its nutritional requirement than the corresponding isolate 7, in nitrogen source, vitamin and trace-metal requirement studies. This strain showed only slightly inhibited growth on the tannic acid medium

The three species L. nuda, L. saeva and C. gambosa may represent three stages in the degradation of dead plant tissues. L. nuda can be considered to occupy a position as a primary saprophytic coloniser within the basidomycete flora. The fungus is able to rapidly colonise partly rotted natural or artificial accumulations of deciduous (commonly beech leaves) or coniferous leaves, and is able to make sufficient growth coupled with tolerance of low temperatures to accumulate sufficient reserves for fruitbody formation prior to the onset of heavy frosts and freezing temperatures. Lignin decomposing strains able to degrade the more resistant plant material in the soil beneath the surface leaf-litter accumulations are suggested to have arisen which colonise organically rich soils. The tolerance of some strains to phenolic materials in plant leaves appear to enable them to colonise the less readily attacked residues (i.e. accumulations of coniferous leaves). Lepista nuda emerges as a genetically adept and nutritionally resourceful fungus able to make use of readily degraded materials (proteins, pectin, starch hemicelluloses) and able to slowly utilise the more resistant fractions (cellulose and lignin).

Lepista saeva typically occurs in pastureland soils and is probably closely associated with the decomposition of roots, stems and leaves within the grass sward. It occasionally inhabits positions

in open copses. L. saeva strains weregenerally slower growing than those of L. nuda on the composted substrate, however, hyphal growth was more dense. This species demonstrated a stronger cellulolytic ability than L. nuda.

Calocybe gambosa similarly to L. saeva is able to form a perennial mycelium which typically gives rise to fairy-rings. It occupies a similar habitat to the latter also occasionally arising within open woodland. Nutritional data indicates that this species is able to strongly decompose lignin, but its cellulolytic capacity is comparatively weak. The ability to breakdown the resistant 'lignin-humin' fraction of soils may enable this fungus to gain access to a variety of nitrogeneous materials, possibly including amino acids, peptides and proteins complexed to the lignin polymer. C. gambosa produced slow growth in pure culture, best growth was obtained in natural media (malt extract, potato dextrose agar) which may be due to a requirement for certain growth factors (high levels of such growth factor(s) are indicated to be present in the leaves of spruce and larch) and for organic nitrogen compounds. These media also have a high carbon:nitrogen ratio.

Mycorrhizal v. Saprophytic status

The nutritional status of mycorrhizal fungi is currently under review (Ferry and Das 1968, Palmer and Hacskaylo 1970, Lewis 1973, Lamb 1974) and also the relative contribution of the commonly associated hymenomycetes to the mycorrhizalflora, has been questioned (Zak and Marx 1964, Lamb and Richards 1970), however, although the nutritional limitations of the mycorrhiza former appear not as restricted as formally considered (Melin 1925) - the possibility of a passive saprophytic existence has been envisaged in the absence of the host plant - the nutritional diversity shown by these three species together with the ecological data are almost certainly sufficient to place them in a solely saprophytic category.

Influence of Oxygen and Carbon Dioxide Tension on Growth

Data from the effect of oxygen concentration on hyphal growth of L. nuda and L. saeva isolates indicated no growth inhibition

until oxygen concentration is reduced to 2-4%. Growth rate is only markedly inhibited at 0.7 to 1.0% at which concentration growth is reduced to approximately half maximal. Oxygen deficiency is therefore unlikely to be a serious hazard for growth of these species in soils and leaf-litter. Oxygen would be limiting only in very heavy compacted or water-saturated soils.

Carbon dioxide at low concentrations (5%) exerted a direct toxic effect on isolates of L. nuda, L. saeva and C. gambosa. Inhibition of growth was most pronounced for two fast growing L. nuda and L. saeva isolates, half maximum growth resulting at values of 2.4% and 5% CO_2 (the growth curve of this L. saeva isolate (6B) was similar to that produced for a second isolate (5) in a repeat experiment). Two slower growing isolates on the synthetic glucose-asparagine medium at 16°C, produced denser colony mats and were less susceptible to inhibition by carbon dioxide. Half maximal growth resulted at 9.5 and 10.6% CO_2 for L. nuda and L. saeva strains respectively. It is suggested that isolates colonising surface leaf-litter (a 'sub-aerial' enviroment), or producing surface growth in light soils and generally producing a more rapidly developing and dispersed mycelium, are relatively more susceptible to the antibiotic action of carbon dioxide than those strains inhabiting deeper soil layers, producing a denser more perennial mycelium. The former instance would be considered to contain L. nuda, the latter L. saeva. For both species, however, the susceptibility to carbon dioxide is considered to be a variable factor within species, relating directly to the ecology of the original isolate.

The growth of the cultivated mushroom, Agaricus bisporus is retarded by carbon dioxide concentrations below 10% (Tschierpe 1960, Nair 1972) although there is some discrepency as to the extent of this inhibition which may be related to strain characteristics or conditions of culture (i.e. humidity, temperature, media composition). Agaricus bisporus is able to thoroughly colonise highly compacted horse manure-straw 'composts' under commercial conditions, without a discernable reduction in growth-rate, this contrasts with poor development of L. nuda and L. saeva strains, following relatively light compaction of

the commercial mushroom growers compost used in solid substrate fruiting trials. Accumulation of respired carbon dioxide to inhibitory levels appears to be the factor responsible.

Carbon dioxide at low concentrations (< 1.0%) markedly inhibited the growth of C. gambosa strain 2. C. gambosa has a high incidence of occurrence on basic soils particularly calcareous clay supporting rough grassland (often grassy banks and roadside verges), it has also been recordedon leaf-litter of birch and ash in open woodland (University of Birmingham, Herbarium records).

Within the aqueous phase of alkaline soils carbon dioxide is largly converted via carbonic acid to bicarbonate which further dissociates at high pH to form carbonate ions (Ponnamperuma 1967, Griffin 1972). The relationship is simply expressed by the equation:

$$H_2O + \underset{\text{gaseous}}{CO_2} \rightleftharpoons H_2CO_3 \overset{+H_2O}{\rightleftharpoons} H_3O^+ + HCO_3^-$$

$$HCO_3^- \overset{+H_2O}{\rightleftharpoons} H_3O^+ + CO_3^{2-}$$

Carbon dioxide may also combine with calcium and magnesium carbonates in aqueous solution (Ponnamperuma 1967).

Dissociation of carbonic acid to form bicarbonate and carbonate ions is directly influenced by hydrogen ion concentration. The importance of the carbon dioxide, carbonic acid, bicarbonate equilibrium to fungal physiology was recognised in early studies (Garrett 1938); more/ recent experimental evidence indicates that bicarbonate is metabolically active at alkaline pH (Griffin 1965, MacCauley and Griffin 1969). The conversion of carbon dioxide to bicarbonate in basic soils results in lower partial pressures of carbon dioxide in these soils relative to acid soils assuming equivalent respiratory rates (Griffin 1972). It may, therefore, be assumed that fungi inhabiting alkaline soils are less exposed to direct toxicity by accumulation of gaseous carbon dioxide through the existence effectively of a bicarbonate sink.

With reservations that the data concerning the effect of carbon dioxide on C. gambosa is limited to a single isolate cultured on an artificial nutrient rich medium (2% malt extract agar), and therefore extrapolations to growth in nature require to be tentative, it is probable that for certain strains growth in nature may be restricted to surface leaf-litter and soil layers, light soils and to alkaline soils due to direct toxicity by carbon dioxide at low concentrations. (0.5 - 1.0%).

Fruitbody Development

A measure of success was achieved in obtaining immature fruitbodies for an isolate of L. nuda in pure culture on a semi-solid medium and fruitbodies of three strains on a non-sterile composted substrate.

Development of fruitbodies in pure culture occurred following 9 weeks maintained at 12°c with a circadian light cycle of 9 hrs light, 15 hrs dark, supplied by fluorescent daylight tubes. Hyphal development over the surface of the agar was promoted by an initial 3 week dark incubation period at 25°c. The culture system consisted of 100 ml quantites of 2% malt extract (Boots Pure Drug Co.,) + 0.1% Yeast extract (Oxoid Ltd.,) solidified by 1.5% agar addition (Oxoid No. 3) forming a c. 2cm deep layer at the base of 250 ml wide mouth Erlenmeyer flasks, plugged with bleached non-absorbent cotton-wool.

Mycelial aggregations representing possible primordia were detected for four strains of L. saeva within the peat chalk capping employed in the growth tubes to prevent desiccation of the agar inoculum.

The L. nuda isolate (9) forming fruitbodies on an agar medium was probably more genetically predisposed towards their development under this system of culture. This phenomenon is characteristic of some strains of Agaricus bisporus which readily produce primordia in pure culture (Hume and Hayes 1972, Angeli-Couvy 1975).

Fruitbody formation probably arises as a direct consequence of a check in hyphal growth (Cochrane 1958). Limitation of growth is

considered to favour formation of reproductive structures (Hawker 1950, Plunket 1953). Work conducted by Hawker with Melanospora destruens indicated 0.5% glucose was optimal, the general finding that oligo-and polysaccharides are more suitable than simple hexoses as sources of carbon for formation of reproductive structures was related to ability of the organism to obtain a constant low level of hexose sugar, this was generally the case for carbon sources which were poorly utilised by the fungus (i.e. cellulose). Ease of phosphorylation was considered to be a critical factor in determining their effect upon fruiting (sucrose was favoured in this respect), the level of phosphorylated hexoses was considered to directly affect respiration rate and hence available energy for chemical synthesis.

High levels of nitrogen generally inhibit fruiting. Formation of reproductive structures does not appear to be favoured by any particular source of nitrogen. The mineral requirement though probably increased is not generally considered to be extended compared to the requirement for vegetative growth, with the possible exception of copper as a component of tyrosinase required for spore pigmentation or calcium acting in an antagonistic capacity. Vitamin nutrition for fruitbody development may be more heterotrophic, there is evidence (Cochrane 1968) of an increased requirement for thiamine and biotin. The suitability of 2% malt extract + 0.1% yeast extract agar upon which fruitbodies were obtained may reflect its ability to fulfil most of these nutritional requirements.

Large numbers of small fruitbodies of L. nuda appear to be able to form where there is only a small amount of substrate provided, this was also observed following colonisation of spent tan bark in plant pots by Constantin and Matruchot (1898). It is not clear whether there is a minimal nutritional requirement for their development, however, further development of more advanced fruitbodies (i.e. those better disposed to obtain in a flow of nutrients from the mycelium) appears to be only possible where the substrate bulk is increased as was the case with the composted substrate in containers or in leaf-piles by Constantin and Matruchot. This mechanism is similar to that first elucidated for Coprinus lagopus (C. cinereus) by Madelin (1956), with L. nuda however, the increased bulk of the sporophore may

delimit a minimum nutritional requirement for primordium formation. This requirement for fruiting on agar may account for a considerably lengthened incubation period prior to fruitbody formation (3 months) or to a minimum substrate level met by the employment of an increaseddepth of agar to that employed for Petri-plate studies. The use of an increased bulk of agar may have a buffer effect against the processes of desiccation and staling experienced with semi-solid media during long term incubation studies. A similar volume of malt extract based agar (Hagem agar modified after Modess, 1941) and flask culture system, has been successfully employed in cultural studies concerning fructification of a number of species of Boletaceae where incubation has continued over a period of several months (Pantidou 1961 a & b, 1962 and 1964).

Requirements for formation of initials or primordia may be quite different to those conditions necessary for further development and expansion of these structures to form mature sporophores. Development may therefore represent two phases, the second developmental phase may have additional requirements for maturation of the hymenium and sporulation.

The requirements for the first phase may be largely nutritional and possibly directly triggered by factors impeding the development of hyphae at the mycelial front. This may be directly related to the ability of the hyphal tips of the lead hyphae to secure a minimum flow of nutrients tosucceeding branch hyphae (Butler 1961).

Translocation of materials to the mycelial front where further hyphal development is prevented may result in accumulation by formation of initials or sclerotia or dissipation in formation of asexual reproductive structures in lower fungi. Where this process is prevented (possibly by adverse physical factors),autolysis with release of stored products into the medium may occur. The processes of primordia formation may be governed by, (a),the ability of the fungus to accumulate reserve materials from the substrate onwhich it is growing, this may be linked to the provision of a slowly metabolisable carbon source; (b),its ability to translocate these, which may be related to its ability to form mycelial

strands; (c), a low concentration of respiratory inhibitors, this may reflect a requirement for a certain low level of carbon dioxide; concentrations of this gas in excess of 0.67% cause inhibition of primordium development in Agaricus bisporus (Long and Jacobs 1974).

Removal of nutrients from the hyphal tips may be effected by colonisation of an inert substrate, removal by metabolism by associated microflora (Long 1968), or use of adsorbing agents such as activated charcoal. Depletion of nutrient status and inactivation may also result from low temperature treatment or mechanical injury.

Isolates forming primordia in pure culture may be those able to form reserve materials on laboratory media, possibly those producing poorest growth on these media (in terms of dry weight) or those less susceptible to inhibition of primordium formation with slightly elevated levels of carbon dioxide.

Physical factors such as light, temperature, humidity and aeration affect further fruitbody development (phase 2) and may determine degree of differentation. Light has been determined to be a requirement for pileus expansion of a wide range of basidiomycetes. A requirement for light was indicated for development of L. nuda sporophores though not determined conclusively.

Low temperature treatment may retard vegetative growth and stimulate primordium development, this has been shown to induce fruitbody development in Collybia velutipes (Kinugawa & Furukawa 1965). This may be an important mechanism in nature for L. nuda and L. saeva acting as a check to further vegetative development of surface hyphae; the accumulation of reserve materials over the previous months would account for sudden appearance of large numbers of sporophores.

ROLE OF BACTERIA IN FRUIT-BODY DEVELOPMENT

The presence of a large bacterial population within the 'casing-layer' of the two phase system of Agaricus bisporus cultivation was first shown by Eger (1963) to be important in the induction of primordium formation. This was demonstrated by employing a divided Petri-plate system, the 'Halbschalen-test' which contained horizontally adjacent equal quantities of a mushroom growers compost (the 'vegetative' substrate) and a peat/chalk 'casing' material (the 'reproductive' support medium) within which there was development of a stimulatory bacterial population. A sterile casing material supported strong vegetative mycelial development and no primodium formation. The inclusion of granulated activated charcoal within the sterile medium promoted low numbers of primordia to develop.

This system represents the transition of a rich to a poor nutrient substrate in the upper section of the cased mushroom bed, though it cannot be considered to duplicate the gradient effects of moisture availability and carbon dioxide concentration which exist within the vertical system.

The work of Hayes, Randle and Last (1969) demonstrated that volatiles produced by the mushroom mycelium (Lockhard and Kneebone 1963) selectively favoured the development of a pseudomonad population comprising Pseudomonas putida and Pseudomonas Group IV. Ps. putida was subsequently shown to possess the ability to induce primordium development in agar culture by employing bacterial inoculation plugs placed at the periphery of a mycelial culture in a simple Petri-plate (single phase system) or on water-agar within a divided Petri-plate (Dual phase system - a refinement of the 'Halbschalen-test') (Hume and Hayes 1972; confirmed - Arrold 1972).

An ability of bacterial metabolites to stimulate primodium formation in A. bisporus was reported by Park and Agnihotri (1969a) However, in later experiments they recorded primoridal inducing responses under aseptic conditions from a range of vitamins (particularly biotin),

plant hormones (gibberellic acid), oxalic acid, autoclaved soil extracts and cell free bacterial filtrates. (Park and Agnihotri 1969b). Eger (1972) reports being unable to confirm the effect of biotin over various concentrations, under a range of septic and aseptic conditions.

The casing layer is considered (Eger 1972) to effectively shield an inducing microbial flora from the antibiotic effect of high concentrations of volatile inhibitor(s) produced by active A. bisporus mycelium, although close contact between inducing bacteria and individual or groups of hyphae was considered as requisit for primordium formation. In experiments using the 'Halbschalen-test' as a model system Pseudomonas putida was shown to only effectively inhibit the growth of certain mushroom strains, which was associated with an inducing effect, when compared with the action of a mixed bacterial casing flora, although the latter also produced variable growth inhibition within narrower limits. Pseudomonad cultures grown on acetone as sole carbon source (considered to be the only abundant volatile during the normal aerated conditions under which primordium formation occurs) when compared with controls lacking acetone, induced higher numbers of primordia. It was concluded that the volatile acetone may play a decisive role in supporting and maintaining a microflora which is stimulatory to primordium formation.

The induction of primordium differentiation and fruitbody development are processes which are dependent on the preliminary formation of an undifferentiated tissue mass, the 'initial'. The development of the 'initial' represents a fundamental and localised re-organisation in the pattern of hyphal branching and hyphal association, resulting in the multiplication and aggregation of hyphae to form a knot of tissue.

The development of the initial and the transition of the initial to the primordium are stages which are still ill-defined. It is probable that the development of the initial may be fairly readily induced by conditions of nutritional stress at the hyphal front, however, its further development into a primordium may be the critical stage which is dependant on the nutritional status of the colony and the existence of favourable external conditions. It may be that bacteria,

activated charcoal, or internal/external conditions causing autolysis, promote development of the initial, its further development may be in response to internal nutrient access though also external conditions of moisture, aeration, temperatureand light.

The formation of the initial and its further expansion and differentiation to form a primordium are therefore considered to be key stages for fruit-body development. These stages may be postulated to result as the culmination of key gene switches where metabolically active substances have acted to repress or derepress the expression of individual gene sequences, these substances arising externally (i.e. CO_2, antibiotics) or internally as a result of physical conditions (i.e. light, temperature, moisture stress).

The role of bacteria is uncertain, their effect has recently been successfully duplicated at fixed carbon dioxide concentrations (100 - 1000 p.p.m.) by the inclusion of activated charcoal in a sterile peat/chalk casing mix (Long and Jacobs 1974). Carbon dioxide is critical in controlling the transition of vegetative to reproductive hyphal growth.

The fruitbody inducing property of bacteria in natural soils and under commercial conditions has been explained by a number of plausible modes of action:

a) metabolism away of substances suppressing initiation produced by the lead hyphae, or the removal of nutrients leaking from the leading hyphae - this is possibly analagous to the action of activated charcoal;
b) the formation of antibiotics or other metabolites which act to suppress mycelial growth and induce development of initials - this may be correlated to the loss of inducing ability of bacterial isolates grown in pure culture over several generations (i.e. loss of an induced enzymic activity) (Eger 1968);
c) direct lysis of fungal hyphae - this has been observed for bacterial isolates by Eger (1968) on hyphae of Lepiota rhacodes and yeast cells.
d) the selected bacterial population may act to make available ferrous iron to the mushroom mycelium within the casing layer - a substantial proportion of the pseudomonad population which becomes established in

the casing layer is able to reduce ferric to ferrous iron (Hayes 1972, Hayes and Nair 1973).

Nutrition of fruitbody formation

A fruitbody inducing hormone has not been conclusively proven to exist; the work of Eger (1970) with Pleurotus has indicated that strongprimordial development can be stimulated by cultures developing from pieces of fruitbody tissue on biomalt agar or by the provision of relatively high levels of certain organic nitrogen sources (i.e. urea or asparagine). The work of Wessels (1967) with Schizophyllum commune has indicated that a constant supply of simple sugars is necessary for pileus development and expansion to occur within the developing primordium.

The processes of fruitbody formation in pure culture on agar media are frequently associated with autolysis coupled with exhaustion of medium components; this complex process renders analysis and comparison difficult, and may further be complicated by unknown conditions of aeration within Petri-plates and flask-culture systems.

Genetic Considerations

Genetical information and detailed information on the life history for these species is lacking.The inability of investigators to readily obtain the fruitbodies (sexual stage) in culture has no doubt accounted for their dismissal as suitable models for genetical studies.

All three species produce four spored basidia. Attempts at obtaining spore germination from spore prints of L.nuda and L. saeva deposited on the surface of malt agar slopes within MacCartney bottles were unsuccessful. All isolates of the species studied were obtained from cap tissue. Difficulties in obtaining germination of L.nuda spores were recorded by Norkrans (1950), however, Constantin and Matruchot (1898) report obtaining cultures of L.nuda from spores which germinated readily on spent tan bark and leaf substrates.

Isolates obtained by tissue culture are fertile dikaryons, this was indicated in these species by the possession of a regular distribution of clamp-connections at individual septa.

With the present knowledge possible mechanisms of out-breeding (bi- or tetrapolar mating type factors, the possession of multiple alleles of incompatibility loci) can only be the subject of speculation. Such information is likely to be compatible with that obtained for Coprinus and Schizophyllum. The use of isolates such as L.nuda (9) could further assist in clarifying these machanisms.

Heterokaryosis is suggested from nutritional data by the divergence between strains in ability to lyse cellulose and strength of the Bavendamm 'oxidase' reaction. Comparison of nutritional data for L.nuda isolates 7 and 8 for vitamin and trace metal requirement and also differential ability to utilise nitrate nitrogen by L.nuda and L.saeva strains is indicative of pronounced heterozygosity which is sexually maintained by mating type machanisms.

Commercial Exploitation

Commercial cultivation is potentially feasible for selected L.nuda strains providing that a means of inducing fruitbody development in a shorter period than three months is found. A cold temperature 'chilling' in specially constructed refrigeration rooms may facilitate the formation of 'timed' flashes of mushrooms. The advantages of cultivation of this species and L.saeva to that of A.bisporus is in an ability to utilise substrates requiring less nitrogenous supplementation and simpler preparation. The growth of single L.nuda and L.saeva isolates on a range of short composted substrates (table 13:1) indicates that good vegetative growth is produced at nitrogen levels of *c.* 1.6 g % N, stronger growth in non-peak heated substrates may indicate that these species favour short composted substrates.

It is probable that substrates comprising wood shavings or sawdust (preferably deciduous) or other sources of cellulosic/hemicellulosic materials such as straw, hay, cereal hulls or corn cobs could be

admixed with high grade nitrogen sources such as cotton-seed meal, soyabean meal or oat meal to produce suitable short composted substrates for commercial exploitation. The scheme envisaged would involve a preliminary mixing and presoaking of these materials over several days following which the material would be subjected to a short peak-heat (to induce a rapid thermophilic fermentation) and terminal pasteurisation.

It is probable that fruitbody yields are directly related to the quality of the nitrogen available, which may arise from microbial nitrogen or the nitrogenous supplements, and the presence of carbohydrate materials which can be readily but selectively utilised by these species such as microbially formed poly-saccharides or hemicelluloses and partly degraded cellulose. Ability to degrade lignin may be an important mechanism is gaining access to these materials by these species. These substrates should selectively favour the natural nutrition of these species the inclusion of a pasteurisation step would serve to eliminate competitors, rapid substrate colonisation should be attainable with a suitably chosen inoculum size.

Lepista nuda makes strong growth and is capable of producing abundant fruiting bodies in nature on partly rotted leaf substrates and therefore should be able to make good utilisation of the cereal straw. Tricholoma personatum as reported by Hard (1961) in America, 'delights to grow where an old sawmill has stood' and further more formed large numbers of fruiting bodies following growth on a compost of old green cobs remaining from a canning factory.

Good growth of L.nuda occurs at lower temperatures than required for A.bisporus, therefore, heating during substrate colonisation may not be necessary. Disadvantages include a longer incubation period required by this species, this disparity may be greatly reduced by substrate selection; and at present delayed fruitbody development. No information is available on yields, yield capacity will be directly related to substrate selection and preparation. Cooling may be required, however, this could be circumvented by cultivation during winter months, and selective use of low outside air temperatures.

A similar system may be employed for L. saeva, growth of this species is, however, less vigorous than that of L. nuda, this difficultly may to some degree be overcome by strain selection.

Lepista nuda has an attractive smell and taste for culinary purposes, it possesses a wide range of essential amino-acids (table 16:0). Mycelium obtained by submerged culture is reported to have a high food value in feeding trials (Bell, Erfle, Spencer & Reusser 1958). Lepista saeva is at present collected and sold in the Midland market towns of Burton-on-Trent, Loughborough, Leicester and Nottingham and is recorded as having been formally sold at Covent Garden market. Both species are sold in quantity in Continental open markets.

CONCLUSIONS

1. The present study formed an investigation into the physiology of three wild, edible, basidiomycete species: Lepista nuda (14 isolates), Lepista saeva (8 isolates), and Calocybe gambosa (5 isolates), including aspects of fruit-body formation. All isolates were obtained by cap-tissue culture. An attempt was made throughout the study to relate observations to the natural ecology of these species. A wholly saprophytic existence possibly as obligate rhizoplane inhabitants, excluding mycorrhizal association, was indicated from the data.

2. All species were routinely cultured and grew well on a malt extract agar, C.gambosa strains demonstrated progressively improved growth with adaptation to this medium during the course of the study. Good growth of L.nuda and L.saeva isolates was obtained on a formulated glucose-asparagine-salts plus thiamine basal liquid medium in stationary culture. Poor growth was shown by C.gambosa strains on this medium without the addition of certain growth factors. Growth on the synthetic medium by this species did not improve over the period of study.

3. Temperature optima of 20°C and 25°C were obtained for L.nuda isolates, 20°C for L.saeva, and 25°C for a single isolate of C.gambosa. Both L.nuda and L.saeva grew at low temperatures 10°C and 4°C (slightly). All species were inhibited at 30°C.

4. Optima in hydrogen ion concentration were determined at pH 7.0 for L.nuda, c.6.0 for L.saeva and 5.5 for C.gambosa. L.nuda exhibited a wider pH tolerance than L.saeva.

5. Investigation of trace metal requirements employing a range of ash additions to a minimal basal salt solution, calcium at relatively high levels of addition was found to be an important component of the complete synthetic medium for attaining high mycelial dry-weights. The probable importance of calcium as a metal-ion buffer is discussed.

6. All strains of L.nuda and L.saeva were heterotrophic for thiamine. Partial heterotrophy was indicated by some strains for biotin, pyridoxine and folic acid. C.gambosa grew slowly in the basal medium with thiamine addition, a partial heterotrophy for a broader range of vitamins by this species was indicated.

7. Optimal C:N ratios for vegetative growth were investigated employing the complete synthetic medium, by alteration of the level of carbon (glucose) maintaining nitrogen level (asparagine conc.,) constant. Growth efficiency (yield) was assessed for four series at increasing concentrations of nitrogen. An optimum C:N ratio of c.21:1 (and an extrapolated optimum nitrogen concentration of 0.1864gN/L) at 28 days incubation was determined for L.nuda and L.saeva. A somewhat higher C:N ratio was indicated as optimal for C.gambosa.

8. Single isolates of these three species were able to utilise a wide range of mono -, di -, tri - and polysaccharides. Combining carbon sources improved utilisation of some polysaccharides. Mannose, glucose, fructose, sucrose, maltose, raffinose, dextrin and starch were particularly favourable sources of carbon and energy.

9. Strains of all three species produced high yields on a broad range of organic, amide, and amino-acid sources of nitrogen. Degree of utilisation of ammonium and nitrate nitrogen was a variable character within species. Growth on these nitrogen sources was correlated with change in hydrogen ion concentration of the culture fluid. No strains tested utilised nitrite nitrogen.

10. A range of alcohols, simple organic acids and fatty acids did not produce significant ($P = 0.05$) yield increases in single L.nuda and L.saeva isolates when added as supplements (50,100 or 500 mg/L) to the complete synthetic medium. Improved yields by C.gambosa

following supplementation was produced by the rapid growth of sectors within the colony.

11. Inclusion of a range of leaf and manure extracts (at 0.5, 1.0 & 3.0 mg/L) into the basal medium produced highly significant growth increases (P = 0.001) of a single C.gambosa isolate in relation to the unsupplemented control value. This was particularly marked with Norway Spruce and European Larch leaf extracts. Significant growth promotion of L.nuda and L.saeva occurred with a considerably narrower range of extracts.

12. Assessment of cellulolytic ability by dissolution of finely milled cellulose fibres, and for polyphenol oxidase activity utilising the classical Bavendamm 'oxidase' test (discolouration of agars incorporating gallic or tannic acids by formation of coloured complexes) - this enzyme activity has been characterised as p-diphenol oxidase (Lindeberg 1946(b), 1948, 1950, Lindeberg et al., 1952(a & b)) - established that L.nuda and L.saeva are able to degrade cellulose and lignin, these enzyme activities were further shown to be a variable characteristic between isolates. C.gambosa demonstrated weak cellulase though high p-diphenol oxidase levels. These activities are discussed in relation to indirect evidence for possession of O-diphenol oxidase activity.

13. Growth rates of a range of L.nuda and L.saeva isolates were assessed through a variety of composted solid substrates in specially constructed cylindrical glass tubes. Extent of stranding was recorded in tubes and following colonisation of compost, peat/chalk casing in flasks. Development of compact nodules of tissue representing possibly fruit-body initials was recorded for four isolates of L.saeva. Strand development, nodulation and growth rate were used as criteria

for selection of strains for fruiting trials on a solid substrate. C.gambosa made poor growth or absence of growth into these composted substrates.

14. Lepista nuda strains forming fruit-bodies on a composted medium plus three strains of L.saeva which produced numerous tissue aggregates in tubes and flasks together with 3 C.gambosa strains, were assessed for their ability to form primordia on a natural agar medium under controlled conditions of lighting and temperature. A study was made of a single L.nuda strain which formed numerous primordia under this system of culture.

15. Fruit-body development by 3 L.nuda isolates was obtained on a commercial mushroom growers compost with a variety of leaf, soil and peat/chalk casing treatments, under artifical conditions. This culture system supported good colonisation of the compost and soil plus peat/chalk casing layers by 3 L.saeva isolates. However, fruit-body development of these isolates did not take place.

16. A diffusion-column technique was employed to assess the effect on colony growth-rate of a range of carbon dioxide and oxygen tensions, above and below atmospheric. Both L.nuda and L.saeva isolates were insensitive to partial pressures of oxygen, descending to 4.0% and below.

Strains of L.nuda, L.saeva and C.gambosa were sensitive to low partial pressures of carbon dioxide ($< 5.0\%$)

The ecological implications (as colonisers of surface leaf-litter and soils) of oxygen tolerance and intolerance to elevated levels of gaseous CO_2, is discussed.

ACKNOWLEDGEMENTS

I should like to thank Dr.W.A.Hayes for his supervision throughout this study; Dr.N.G.Nair, Department of Agriculture, Ryadalmere, N.S.W., for assistance with the gas-diffusion column; and Dr.R.Watling, Royal Botanic Gardens, Edinburgh, for encouragement and guidance, particularly concerning taxonomy of the Higher Fungi, and for provision of several isolates.

The assistance of the members of the Warwickshire Fungus Survey in obtaining isolates, and by Dr.G.M.Butler, Department of Botany, University of Birmingham, in co-ordinating these efforts, is gratefully acknowledged.

I should also like to thank postgraduate students within the Mushroom Science Laboratory, Richard Steane, Rosemarie Pearson, Nahida Haddad and Kenneth Jakeman (technician) and in the adjoining Fermentation Laboratory, Stephen Pannell and Robert Spensley, for companionship, assistance, discussion and criticism during the course of this study.

Appreciation is also extended for technical assistance provided by members of the Communications Media Department, Mr Johnston, Roger Banks and Roger Howes.

I would like, finally, to thank the Science Research Council for the postgraduate award, which made this study possible.

A P P E N D I X

APPENDIX: INDEX TO TABLES

TABLES 2.8 &9 Phosphate Buffer Tables.
(Lindeberg 1939)

BUFFER CONCENTRATION		THEORETICAL pH.	EXPERIMENTALLY OBTAINED VALUES (of Lindeberg)
ml M/15 KH_2PO_4	ml N/10 HCl		
75	75	2.0	2.1
129	21	3.0	3.2
146	4	4.0	4.1
ml M/15 KH_2PO_4	ml M/15 Na_2HPO_4		
149.5	0.5	5.0	5.2
131.5	18.5	6.0	6.1
55	95	7.0	7.0
3	147	8.0	8.2
ml M/15 K_3PO_4	ml M/15 NaH_2PO_4		
13.5	136.5	9.0	8.8

Revised Buffer Table (of Modess 1941)

ml M/15 KH_2PO_4	ml N/10 HCl	THEORETICAL pH	Author's Values obtained with Glucose–Asparagine synthetic Medium
75	75	2.0	2.35
110	40	2.5	
130	20	3.0	3.1
140	10	3.5	
145	5	4.0	3.7
147	2.5	4.5	
149	0.5	5.0	4.32
ml M/15 KH_2PO_4	ml M/15 Na_2HPO_4		
145	5	5.5	
130	20	6.0	5.93
105	45	6.5	
60	90	7.0	6.9
20	130	7.5	
	150	7.8	7.73

TABLE 3.1-3 EFFECT OF TEMPERATURE ON GROWTH
Colony extension (cm) at 21 days incubation
(C.gambosa 21 & 42 days incubation).

TEMPERATURE (°C)	LEPISTA NUDA				LEPISTA SAEVA			CALOCYBE GAMBOSA	
	1	247.69	5	8	1	248.69	6	2	
4	0.8	0.8	1.6	1.5	1.4	1.4	1.3	0	0
10	1.7	3.5	4.7	4.3	4.9	4.4	4.6	1.2	1.6
15	3.7	4.5	6.5	5.9	6.7	5.9	5.8	2.9	6.3
20	5.7	5.8	7.0	6.5	8.5	6.5	6.6	3.9	6.8
25	7.0	6.8	6.2	4.7	7.3	5.3	6.0	4.8	8.5
30	0	0	0	0	0	0	0	0	0

TABLE 2.1-7 EFFECT OF HYDROGEN ION CONCENTRATION ON GROWTH (21 days incubation)

pH THEOR-ETICAL	pH ACTUAL	LEPISTA NUDA			LEPISTA SAEVA		CALOCYBE GAMBOSA	
		6 mg	7 mg	8 mg	248.69 mg	5 mg	6 mg	1 mg
3.0	3.1						22.0 (3.5)	
4.0	3.7	46.0 (5.22)		45.0 (4.9)			35.5 (5.65)	
4.5	4.32	73.2 (5.02)	38.1 (3.5)	60.0 (5.03)	48.0 (4.65)	35.1 (4.35)	52.7 (5.57)	24.2 (5.2)
5.0	5.0	85.0 (5.02)	117.0 (3.35)	84.0 (5.04)	154.9 (4.75)	83.6 (4.38)	87.6 (5.38)	40.7 (5.0)
6.0	5.93	103.0 (4.77)	138.5 (4.55)	82.0 (5.05)	223.0 (5.6)	183.7 (5.1)	74.3 (5.9)	18.2 (5.85)
7.0	6.9	108.1 (5.72)	190.5 (5.45)	97.5 (5.4)	124.0 (6.75)	102.0 (6.56)	45.1 (6.1)	26.0 (7.08)
8.0	7.73	75.6 (7.3)	80.5 (6.6)	64.0 (7.0)	83.0 (7.3)	50.0 (5.65)	39.0 (6.68)	

( ) pH culture fluid, post incubation.

TABLE 4.1—2

ASH OR METAL ION ADDITION	Lepista nuda		Lepista saeva	
	7 mg	8 mg	6B mg	5 mg
BASAL SOLUTION	93.8 (4.75)	119.3 (5.1)	42.6 (5.8)	44.0 (4.75)
BASAL + BEECH LEAF LITTER ASH	143.7* (6.0)	133.2 (5.75)	92.5** (6.05)	139.6 (6.0)
BASAL + CHESTNUT LEAF LITTER ASH	166.5** (5.85)	95.7 (6.1)	113.0** (5.95)	132.8** (5.45)
BASAL + LARCH LEAF LITTER ASH	166.6** (5.45)	100.1 (5.8)	154.0** (5.7)	122.3** (4.75)
BASAL + HORSE MANURE ASH	194.5** (5.75)	110.6 (5.95)	115.9** (5.7)	116.8** (5.2)
BASAL + MALT EXTRACT ASH	158.6** (5.7)	88.5 (6.15)	63.6* (5.6)	115.8** (5.3)
BASAL + YEAST EXTRACT ASH	147.9* (5.4)	143.9 (5.1)	127.7** (5.9)	63.7* (4.55)
BASAL + SYNTHETIC MINERAL SOLUTION	63.5 (4.45)	141.6 (5.5)	83.6** (5.6)	40.2 (4.6)
BASAL + ZINC	62.8 (4.35)	115.7 (5.1)	64.3* (5.75)	37.4 (4.4)
BASAL + ZINC + CALCIUM	87.1 (4.4)	89.4 (5.2)	98.8** (5.15)	50.3 (4.3)
COMPLETE SYNTHETIC MEDIUM	208.7** (4.9)	108.5 (5.4)	156.4** (5.8)	158.7** (4.95)

* SIGNIFICANT (P = 0.01)

** SIGNIFICANT (P = 0.001)

Increase in yield to that attained on Basal solution.

( ) pH value of culture fluid, post-incubation.

TABLE 5.1-3

VITAMIN SUPPLEMENT	LEPISTA 7 mg	NUDA 8 mg	LEPISTA 5 mg	SAEVA 6B mg	CALCOCYBE 1 mg	GAMBOSA 2 mg
YEAST EXTRACT	146.6	101.2	148.0	189.4	10.3	12.0
COMPLETE VITAMIN SOLN; (CV)	133.7	76.4	121.0	124.8	3.6	4.0
CV - PYRIDOXINE	99.4*	68.0	91.5*	85.3*	0.8	1.0
CV - THIAMINE	60.8*	44.3*	39.1*	27.2*	0.6	0.7
CV - RIBOFLAVIN	133.3	94.6	107.3	111.0	1.0	4.4
CV - CALCIUM PANTOTHENATE	124.6	84.9	121.0	99.2*	0.6	1.2
CV - NIACIN	122.2	83.3	121.7	95.9*	0.6	0.6
CV - BIOTIN	107.*	79.7	86.4*	79.6*	0.7	0.6
CV - p - AMINO-BENZOIC ACID	105.9*	83.5	103.6	94.6*	1.0	0.7
CV - FOLIC ACID	92.7*	97.0	86.0*	86.2*	0.8	0.6
VITAMIN - FREE CONTROL	52.3*	37.9*	26.1*	20.7*	1.7	2.7

* Significantly lower (P = 0.001) than yield on complete Vitamin solution.

TABLE 6.1 EFFECT OF C/N RATIO ON GROWTH

CALOCYBE GAMBOSA (28 days incubation)

C/N RATIO	MYCELIAL YIELD (^{mg}/Dry Wt) NITROGEN LEVEL (gN/L)			TERMINAL pH NITROGEN LEVEL (gN/L)		
	0.0932	0.3728	0.5592	0.0932	0.3728	0.5592
9:1	17.2	20.0	25.7	6.25	6.1	6.0
12:1	20.1	23.8	33.0	6.15	6.4	6.0
15:1	21.5	26.4	35.9	6.18	6.02	5.7
18:1	22.8	27.4	38.0	6.31	6.05	5.3
21:1	24.0	28.1	39.0	6.22	5.8	5.3
25:1	24.6	34.1	41.8	6.22	5.85	5.05
28:1	25.9	35.9	44.2	6.25	5.8	4.7

TABLE 6.0 PROPORTIONS BY WEIGHT OF GLUCOSE AND ASPARAGINE COMPONENTS AT INDIVIDUAL C/N RATIOS

1g GLUCOSE = 0.4g CARBON 1g ASPARAGINE = 0.32g CARBON ($NH_2CO.CH_2.CH(NH_2)(COOH.H_2O)$)
0.1864g NITROGEN

C/N RATIO	NITROGEN LEVEL (g N/LITRE)			
	0.0932	0.1864	0.3728	0.5592
	GRAMS GLUCOSE PER LITRE			
9:1	2.1	4.2	8.4	12.6
12:1	2.8	5.6	11.2	16.8
15:1	3.5	7.0	14.0	21.0
18:1	4.2	8.4	16.8	25.2
21:1	4.9	9.8	19.6	29.4
25:1	5.9	11.7	23.4	35.1
28:1	6.5	13.0	26.0	39.0

TABLE 6.2 EFFECT OF C/N RATIO ON GROWTH

LEPISTA NUDA 7 (28 days incubation)

C/N RATIO	MYCELIAL YIELD ($^{mg}/_{Dry\ Wt}$) NITROGEN LEVEL (gN/L)				TERMINAL pH NITROGEN LEVEL (gN/L)			
	0.0932	0.1864	0.3728	0.5592	0.0932	0.1864	0.3728	0.5592
9:1	30.9	68.8	121.5	191.0	6.15	6.2	5.95	7.1
12:1	46.4	76.6	118.1	138.6	5.8	5.7	4.35	4.4
15:1	63.2	84.7	110.7	109.9	5.6	5.25	4.0	3.82
18:1	68.8	104.5	120.1	129.1	5.65	4.8	3.8	3.8
21:1	77.3	114.2	139.6	133.5	5.4	4.45	3.75	3.8
25:1	94.7	127.6	157.4	150.7	4.95	3.85	3.5	4.0
28:1	101.1	81.5	165.4	154.0	4.8	3.5	3.85	4.2

LEPISTA SAEVA 6A

9:1	43.6	75.6	162.6	246.8	6.3	6.7	6.7	6.6
12:1	61.6	117.9	190.5	277.6	6.1	6.32	5.2	5.68
15:1	78.0	142.6	211.8	305.3	5.95	5.93	4.05	4.15
18:1	88.1	162.0	288.0	321.5	5.9	5.8	4.1	4.25
21:1	101.7	193.8	303.5	330.4	5.9	5.6	3.9	3.9
25:1	122.3	147.3	285.3	367.9	5.65	4.1	3.85	4.2
28:1	120.8	136.5	244.9	280.4	5.05	3.93	3.8	4.2

TABLES 6.3-4 EFFECT OF C/N RATIO ON GROWTH

LEPISTA NUDA 7.

0,1864 gN/L SERIES

C/N RATIO	g GLUCOSE PER LITRE	MYCELIAL YIELD (mg/DryWt) DAYS (OF HARVEST) 7	14	21	28	TERMINAL pH DAYS (OF HARVEST) 7	14	21	28
9:1	4.2	21.4	24.9	29.4	38.1	5.92	5.92	5.9	5.3
12:1	5.6	23.1	32.0	37.6	47.9	5.92	5.85	5.6	5.05
15:1	7.0	21.5	31.0	43.6	59.9	5.93	5.82	5.5	4.9
18:1	8.4	26.5	35.8	46.6	62.4	5.9	5.75	5.6	5.3
21:1	9.8	26.0	36.4	52.5	77.0	5.9	5.7	5.53	5.13
25.:1	11.7	26.5	41.9	54.7	71.0	5.9	5.8	5.58	5.2
28:1	13.0	31.4	44.6	62.5	69.0	5.9	5.73	5.55	5.1

0.5592 gN/L SERIES

9:1	12.6	30.7	55.5	74.5	62.1	5.9	5.73	5.8	5.65
12:1	16.8	37.5	65.2	80.4	99.6	5.9	5.6	5.7	5.97
15:1	21.0	44.2	65.2	101.5	116.9	5.9	5.7	5.83	5.96
18:1	25.2	44.7	85.0	103.8	136.0	5.9	5.5	5.75	5.87
21:1	29.4	51.5	94.7	117.5	141.0	5.82	5.45	5.55	5.87
25:1	35.1	51.9	78.8	103.2	149.0	5.8	5.68	5.5	5.76
28:1	39.0	49.6	67.2	93.6	171.0	5.8	5.65	5.55	5.7

TABLES 6.5-6 EFFECT OF C/N RATIO ON GROWTH

LEPISTA SAEVA 6B

0.1864 gN/L SERIES

C/N RATIO	g GLUCOSE PER LITRE	MYCELIAL YIELD (mg/DryWt) DAYS (OF HARVEST) 7	14	21	28	TERMINAL pH DAYS (OF HARVEST) 7	14	21	28
9:1	4.2	25.2	51.6	82.4	80.4	6.0	5.95	6.0	6.7
12:1	5.6	24.5	64.7	103.8	107.6	5.97	5.95	6.0	6.45
15:1	7.0	27.5	74.3	124.7	127.0	6.0	5.9	5.95	6.1
18:1	8.4	27.2	71.3	133.8	147.0	6.0	5.9	5.7	5.8
21:1	9.8	28.2	73.8	142.4	167.1	5.95	5.9	5.42	5.64
25:1	11.7	26.1	88.7	146.3	160.8	5.95	5.88	5.6	4.65
28:1	13.0	23.2	65.9	112.3	110.0	5.95	5.85	5.75	3.5

0.5592 gN/L SERIES

9:1	12.6	23.9	72.2	140.8	208.6	5.95	5.76	4.9	5.75
12:1	16.8	25.8	81.7	167.9	228.0	5.95	5.7	4.55	3.75
15:1	21.0	31.4	101.1	184.6	246.8	5.95	5.72	5.1	3.9
18:1	25.2	33.0	100.4	190.2	254.0	5.9	5.65	4.95	4.12
21:1	29.4	35.0	100.0	191.4	263.0	5.85	5.65	5.25	4.1
25:1	35.1	34.5	101.9	219.4	284.0	5.8	5.62	5.21	4.18
28:1	39.0	32.5	98.7	200.0	275.0	5.75	5.62	5.3	4.35

TABLE 7.1–3

CARBON SOURCES	LEPISTA NUDA 7 mg	LEPISTA SAEVA 6B mg	CALOCYBE GAMBOSA 2 mg
D – XYLOSE	4.3	2.0	14.5
D – RIBOSE	28.7	50.9	6.8
L – ARABINOSE	3.9	2.4	9.6
L – SORBOSE	2.2	0.9	3.5
D – GLUCOSE	212.7	345.0	18.2
D – FRUCTOSE	198.8	302.0	19.8
D – MANNOSE	203.5	342.5	18.0
D – GALACTOSE	97.8	20.2	17.8
SUCROSE	243.5	307.6	12.6
MALTOSE	244.9	269.3	20.9
CELLOBIOSE	188.5	264.1	16.3
LACTOSE	90.5	85.3	13.5
TREHALOSE	224.2	223.7	10.3
D – MELIBIOSE	46.2		
RAFFINOSE	263.3	31.1	11.9
DEXTRIN	286.0	308.8	7.5
STARCH	160.2	296.4	9.3
GLYCOGEN	201.0	278.0	7.4
PECTIN	29.2	1.5	6.7
INULIN	120.4	240.2	14.8
XYLAN	89.7	251.1	7.2
∝- CELLULOSE	80.3	85.0	5.9
L – RHAMNOSE	15.4	9.8	4.5
D – DULCITOL	42.7	10.1	7.5
D – MANNITOL	81.4	13.4	12.0
D – SORBITOL	136.8	68.7	

TABLE 7.1-3 cont:

CARBON SOURCES	LEPISTA NUDA 7 mg	LEPISTA SAEVA 6B mg	CALOCYBE GAMBOSA 2 mg
GALACTOURONIC ACID	24.1	24.1	3.4
CITRIC ACID	4.7	1.5	3.1
DL - MALIC ACID	5.2	6.9	3.2
D - TARTARIC ACID	7.7	4.0	3.7
SUCCINIC ACID	11.0	10.1	3.0
CALCIUM OXALATE	3.3	0.2	1.2
CONTROL	3.6	4.4	3.3
L.S.D. (Inclusive of combined carbon sources)	21.6	17.7	2.7

TABLE 8.1-3

NITROGEN SOURCES	LEPISTA 7 mg	NUDA 8 mg	LEPISTA 6B mg	SAEVA 5 mg	CALOCYBE 1 mg	GAMBOSA 2 mg
PEPTONE	169.1 (4.75)	188.8 (4.95)	106.4 (4.9)	122.1 (4.65)	9.7 (5.55)	18.7 (5.75)
CASEIN HYDROLYSATE	174.1 (4.55)	140.4 (5.4	158.8 (5.65)	214.5 (5.2)	25.7 (5.95)	15.7 (5.9)
UREA	197.6 (6.6)	155.6 (6.85)	61.3 (7.15)	81.8 (6.95)	4.4 (7.65)	1.6 (7.6)
ASPARAGINE	208.7 (4.9)	108.9 (5.4)	156.4 (5.8)	158.7 (4.95)	19.3 (6.25)	1.9 (6.2)
PROPIONAMIDE	60.6 (5.6)	92.2 (6.9)	54.7 (6.0)	79.2 (6.45)	1.2 (5.95)	3.6 (5.95)
AMMONIUM BICARBONATE	178.6 (4.25)	150.2 (6.25)	128.3 (6.5)	110.6 (4.7)	1.4 (7.35)	1.9 (7.45)
AMMONIUM TARTRATE	139.7 (4.55)	82.2 (4.9)	69.9 (4.9)	74.3 (4.6)	9.5 (5.5)	6.4 (5.85)
SODIUM NITRATE	153.7 (5.7)	66.9 (5.65)	24.3 (5.95)	133.0 (6.55)	4.1 (5.8)	2.3 (5.85)
SODIUM NITRITE	1.3 (6.0)	1.2 (6.05)	0.2 (6.1)	1.7 (6.05)	0.7 (6.1)	1.0 (6.1)
POTASSIUM NITRATE	205.5 (5.95)	61.2 (5.75)	35.2 (6.15)	141.4 (6.8)	7.8 (5.8)	3.8 (5.9)
CONTROL	4.5 (5.5)	4.1 (6.0)	0.7 (5.9)	3.5 (5.85)	3.6 (5.9)	1.7 (5.95)

( ) pH value of culture fluid, post incubation.

TABLE 8.4-6

AMINO-ACIDS	LEPISTA NUDA 7 mg	LEPISTA SAEVA 6B mg	CALOCYBE GAMBOSA 2 mg
GLYCINE	212.6 (4.3)	245.2 (5.2)	4.1 (5.65)
DL - ∝ ALANINE	207.9 (4.8)	170.0 (5.5)	4.4 (5.55)
DL - VALINE	165.8 (4.3)	44.1 (5.1)	11.1 (5.23)
L - LEUCINE (+ISOLEUCINE)	220.6 (3.7)	80.6 (4.1)	2.5 (5.85)
L - CYTEINE	42.7 (4.58)	31.9 (5.4)	3.4 (5.41)
L - CYSTINE	29.9 (5.18)	12.6 (4.2)	5.7 (5.83)
DL - METHIONINE	99.7 (4.3)	29.6 (4.0)	6.5 (5.55)
L - ASPARTIC ACID	270.5 (5.55)	222.2 (7.2)	5.6 (5.94)
L - GLUTAMIC ACID	193.6 (6.55)	243.2 (7.48)	6.3 (5.74)
L - LYSINE - HCL	3.1 (5.5)	3.6 (5.65)	2.8 (5.75)
L - SERINE	188.8 (4.6)	247.6 (4.6)	5.8 (5.5)
L - THREONINE	125.8 (4.92)	39.7 (4.95)	5.2 (5.75)
L - ARGININE	156.8 (3.62)	73.2 (3.65)	5.4 (4.62)
L - HISTIDINE	3.8 (5.8)	1.6 (5.8)	3.0 (5.92)
DL-β-PHENYLALANINE	7.3 (5.18)	12.1 (4.6)	9.0 (5.8)
L - TYROSINE	11.0 (5.6)	1.4 (5.7)	4.7 (5.75)
L - TRYPTOPHAN	17.4 (4.9)	22.3 (5.34)	5.8 (5.6)
L O PROLINE	2.0 (5.7)	1.2 (5.85)	3.5 (5.9)
L - HYDROXY-PROLINE	0.8 (5.68)	0.2 (5.85)	4.6 (5.9)
ASPARAGINE	212.8 (4.2)	335.6 (4.94)	4.3 (5.43)
GLUTAMINE	193.2 (3.47)	67.3 (3.7)	6.7 (5.13)
AMINO-ACID MIX	170.2 (4.07)	71.8 (4.08)	17.6 (5.37)
CASEIN HYDROLYSATE	216.8 (3.95)	313.6 (5.2)	11.3 (5.35)
CONTROL	1.6 (5.82)	0.7 (5.88)	5.1 (5.9)

( ) pH culture fluid, post incubation.

TABLE 10.1

EXTRACTED MATERIAL	EXTRACT CONCENT-RATION (mg/ml)	LEPISTA NUDA 7 mg	LEPISTA SAEVA 6B mg	CALOCYBE GAMBOSA 2 mg
PIG MANURE	0.5	164.8	248.5	28.3
	1.0	237.8*	200.0	32.3
	3.0	232.8	179.7	31.3
BULLOCK MANURE	0.5	238.8*	237.7	47.8**
	1.0	247.0*	251.2	36.6
	3.0	279.0**	236.0	48.1**
HORSE MANURE	0.5	172.8	241.3	35.5
	1.0	203.2	230.8	79.6**
	3.0	233.6	172.0	102.8**
S. CHESTNUT LEAVES	0.5	207.3	247.0	51.0**
	1.0	177.2	282.0**	46.0**
	3.0	197.0	239.3	41.8*
BEECH LEAVES	0.5	254.5**	258.0	95.3**
	1.0	225.3	272.3*	39.3*
	3.0	277.9**	184.9	36.5
S. BIRCH LEAVES	0.5	225.9	86.7	39.1*
	1.0	263.7**	121.5	51.6**
	3.0	288.0**	147.2	40.0*
OAK LEAVES	0.5	222.0	164.2	46.2**
	1.0	263.5**	164.3	45.1**
	3.0	212.0	202.5	60.6**
C. BEECH LEAVES	0.5	194.4	175.0	45.3**
	1.0	144.6	209.4	42.3*
	3.0	61.8	82.8	7.2
L. CYPRESS LEAVES	0.5	211.8	231.8	44.5**
	1.0	204.5	207.8	36.8
	3.0	179.0	189.7	28.2
PINE LEAVES	0.5	148.6	131.0	41.5*
	1.0	147.0	131.0	37.2
	3.0	142.5	133.0	52.0**
SPRUCE LEAVES	0.5	154.2	233.7	128.7**
	1.0	196.9	220.7	118.3**
	3.0	235.8	171.5	126.0**
LARCH LEAVES	0.5	157.0	279.1*	107.0**
	1.0	218.4	275.3*	104.0**
	3.0	177.6	227.5	113.0**
CONTROL		199.2	213.9	17.9

* SIGNIFICANT (P=0.01)
** SIGNIFICANT (P=0.001)
} INCREASE IN YIELD TO THAT ATTAINED ON THE CONTROL.

TABLE 15.1-2 EFFECT OF OXYGEN CONCENTRATION ON COLONY GROWTH RATE.

0 - 21.0% OXYGEN.

OXYGEN CONCENTRATION %	LEPISTA NUDA 7 mm/day	LEPISTA SAEVA 5 mm/day	LEPISTA NUDA 247.69 mm/day	LEPISTA SAEVA 248.69 mm/day
0.30	0.42	0.32	0.3	0.3
1.25	3.39	1.79	2.11	1.41
1.76	3.54	1.88	2.22	1.44
2.27	4.10	1.93	2.27	1.56
2.79	4.16	2.16	2.20	1.62
3.77	4.31	2.19	2.13	1.67
4.73	4.46	2.26	2.06	1.77
5.75	4.42	2.24	2.24	1.76
6.75	4.63	2.25	2.16	1.70
7.76	4.56	2.35	2.18	1.75
8.75	4.56	2.32	2.11	1.95
10.72	4.71	2.32	2.24	2.10
12.69	4.60	2.28	2.20	1.95
14.69	4.63	2.35	2.34	2.14
16.69	4.54	2.37	2.30	2.10
18.69	4.64	2.37	2.24	2.06
21.00	4.50	2.41	2.14	2.05

TABLE 15.3 EFFECT OF CARBON DIOXIDE CONCENTRATION ON COLONY GROWTH RATE.

0 - 12.0% CO_2

CARBON DIOXIDE CONCENTRATION %	LEPISTA NUDA 247.69 mm/day	LEPISTA SAEVA 248.69 mm/day
0.69	1.91	1.52
1.06	2.06	1.50
1.43	1.76	1.50
1.81	1.77	1.38
2.51	1.80	1.35
3.21	1.64	1.30
3.95	1.67	1.24
4.69	1.60	1.20
5.41	1.67	1.07
6.12	1.65	1.06
7.55	1.32	1.07
8.98	1.03	1.02
10.43	0.77	0.82
11.87	0.83	0.40

TABLE 15.4-5 EFFECT OF CARBON DIOXIDE CONCENTRATION ON COLONY GROWTH RATE.

0 - 5% CO_2

CARBON DIOXIDE CONCENTRATION %	LEPISTA NUDA 7 mm/day	LEPISTA SAEVA 6B mm/day	LEPISTA SAEVA 5 mm/day	CALOCYBE GAMBOSA 1 mm/day
0	4.52	2.10	2.2	1.44
0.23	4.72	2.60	2.2	1.24
0.35	4.72	2.60	2.1	0.79
0.48	4.60	2.60	2.1	0.76
0.60	4.40	2.56	2.1	0.68
0.84	4.20	2.44	2.1	0.68
1.07	3.96	2.40	2.1	0.65
1.32	3.56	2.28	1.89	0.65
1.56	3.48	2.16	1.89	0.65
1.80	3.00	2.00	1.89	0.62
2.04	3.00	1.96	1.72	0.62
2.52	2.24	1.80	1.61	0.60
3.00	1.56	1.68	1.5	0.41
3.48	1.52	1.60	1.5	0.35
3.96	1.12	1.52	1.4	0.35
4.49	1.10	1.40	1.16	0.35
5.00	0.92	1.32	1.16	0.32

Figure 15.6 Calibration for Carbon Dioxide concentration gradient within Diffusion-column, adjusted for values of 1,0% & 0% within the individual displacement chambers (readings obtained with Fisons Mini Gas-Chromatograph)

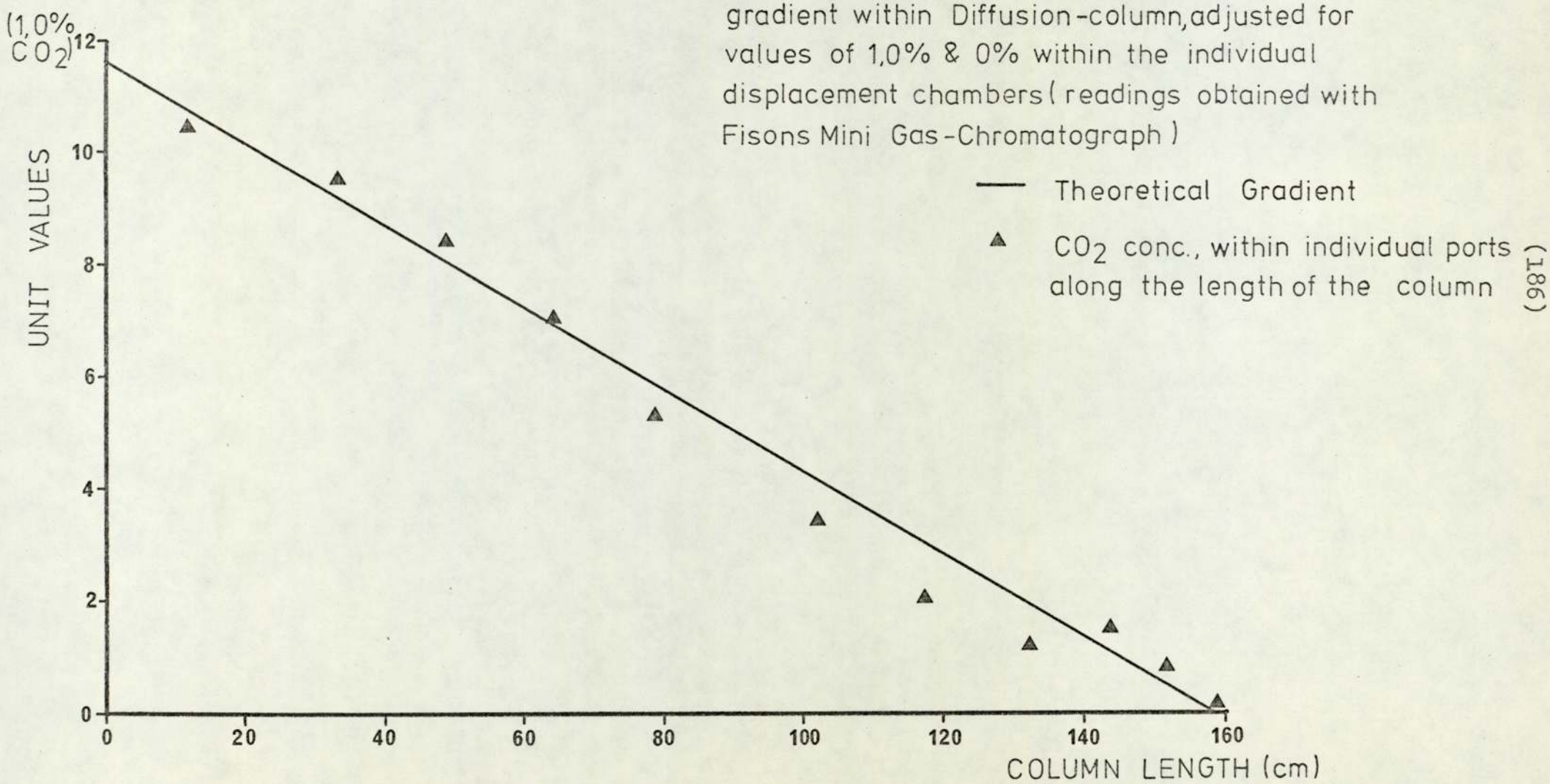


TABLE 16.0

AMINO-ACID ANALYSIS *Lepista nuda* SPOROPHORES †

AMINO-ACID	ISOLATE 8		ISOLATE 9	
	% DRY-WEIGHT	% TOTAL AMINO-ACIDS	% DRY-WEIGHT	% TOTAL AMINO-ACIDS
GLUTAMIC ACID	3.58	17.9	4.23	19.7
ASPAR TIC ACID	1.98	9.9	2.36	11.0
ALANINE	1.73	8.6	1.73	8.1
LEUCINE	1.4	7.0		
THREONINE	1.39	6.9	1.55	7.2
ARGININE	1.30	6.5	1.45	6.8
SERINE	1.28	6.4	1.38	6.4
VALINE	1.21	6.0	1.31	6.1
LEUCINE			1.17	5.4
LYSINE	1.20	6.0	1.11	5.2
GLYCINE	1.02	5.1	1.11	5.2
PROLINE	0.99	4.9	1.09	5.1
PHENLYALANINE	0.97	4.8	0.95	4.4
ISOLEUCINE	0.90	4.5	0.95	4.4
HISTIDINE	0.49	2.4	0.53	2.5
TYROSINE	0.39	1.9	0.39	1.8
METHIONINE	0.22	1.1	0.17	0.8
TRYPTOPHAN	*		*	
CYSTINE	*		*	
TOTAL	20.1		21.5	

* DESTROYED DURING ANALYSIS

† SPOROPHORES HARVESTED AS 'FLATS'.

TABLE 17.0

British Oxygen Co., Ltd., Normal Product specification for Commercial Compressed Oxygen, Nitrogen and Carbon Dioxide.

1. OXYGEN

Oxygen	99.7% (on dry gas)
Nitrogen) Argon)	< 3000 v.p.m.
Carbon Dioxide	< 2 v.p.m.
Carbon Monoxide	< 1 v.p.m.
Hydrocarbons as CH_4	< 20 v.p.m.
Water Vapour	< 50 v.p.m.

2. NITROGEN

Nitrogen	99.7%
Oxygen	< 3000 v.p.m.
Carbon Dioxide	< 1 v.p.m.
Carbon Monoxide	< 1 v.p.m.
Hydrocarbons as CH_4	< 1 v.p.m.
Argon	included in Nitrogen
Moisture	< 200 v.p.m.

3. CARBON DIOXIDE

Inert Gases (insol. in KOH soln.,)	100 pp.m v/v
Hydrocarbons as CH_4	10 pp.m w/w
Oil	2 pp.m w/w
Sulphur (inorganic)	0.5p.p.m w/w
Halogens	1 p.p.m w/w
Moisture	10 p.p.m w/w

TABLE 18.1 (A&B) Natural/Semi-synthetic Media.

A. Modified Cellulose Medium (employed in Petri-plates).

L- asparagine	1g.
Cellulose (4%) suspension	250 ml.
(72HR BALL-MILLED WHATMAN CFII)	
M/15 KH_2PO_4	131.5 } pH 6.0
M/15 Na_2HPO_4	18.5 }
$MgSO_4.7H_2O$	0.5g
Ferric Citrate	5 mg.
(1% soln. in Citric Acid)	
NaCl	0.1g
$ZnSO_4.7H_2O$	4.4mg.
$CaCl_2.2H_2O$	0.1g
'Start glucose'	50mg.
Oxoid Yeast Extract	50mg.
Distilled Water	to 1000 ml.
Oxoid Agar No.3	15g.

B. Cellulose Agar (Eggins & Pugh 1962) (employed in cellulose columns).

L - asparagine	0.5g.
Cellulose suspension (4%)	250 ml.
KH_2PO_4	1g.
$(NH_4)_2SO_4$	0.5g.
KCl	0.5g.
$MgSO_4.7H_2O$	0.2g.
$CaCl_2.2H_2O$	0.1g.
Oxoid Yeast Extract	0.5g.
Distilled Water	to 1000 ml.
Oxoid Agar No.3	20g.

TABLE 18.2 (A B&D) Natural/Semi-synthetic Media.

A. Lutz Malt Agar (Singer 1962).

Malt Extract (Boots Pure Drug Co.)	10g.
NH_4NO_3	1g.
$NH_4H_2PO_4$	1g.
$MgSO_4.7H_2O$	0.1g
Ferric Sulphate	0.1g.
$MnSO_4.7H_2O$	0.05g
Distilled water	to 1000 ml.
Oxoid Agar No.3	15g.

B. Treschow Wort Agar.(Treschow 1944).

Glucose	10g.
L-asparagine	1g.
KCl	0.2g.
$MgSO_4.7H_2O$	0.2g.
$CaCl_2.2H_2O$	0.2g.
$FeCl_3.6H_2O$ (1% soln.)	1 ml.
M/15 Na_2HPO_4	75 ml.
M/15 KH_2PO_4	15 ml.
12^o Wort	10 ml.
(pH adjusted to 6.8)	
Distilled water	900 ml.
Oxoid Agar No.3	15g.

Precautions to avoid precipitation of insoluble salt complexes involve adding calcium and ferric chlorides plus phosphates separately to a cooled medium.

D. COMPOSITION OF PEAT/CHALK 'CASING' MIXTURE.

	$V/_V$	$W/_W$
Ground Chalk	1	1
Medium Grade Sphagnum Peat	18	2.3
Water	8	5

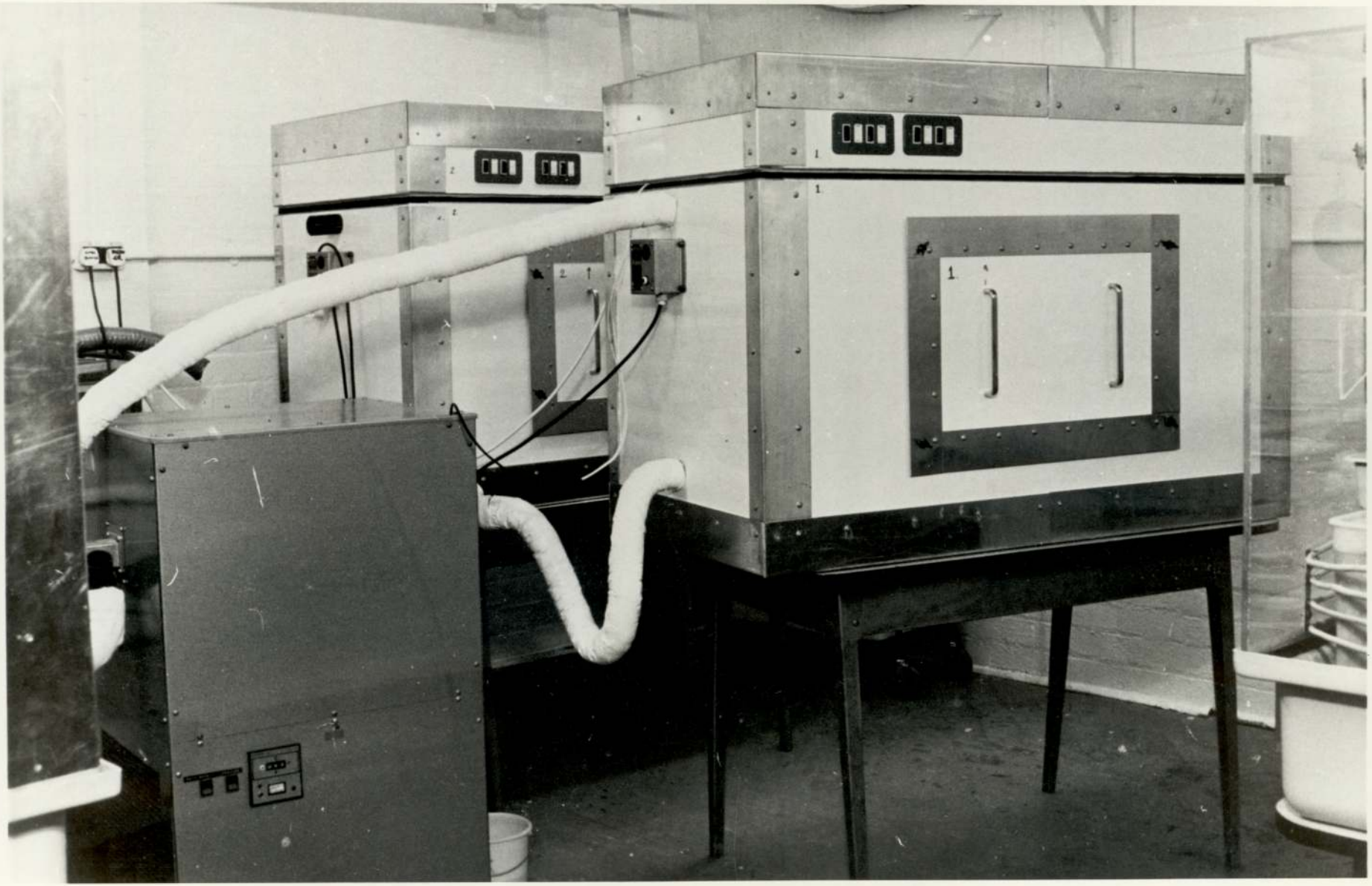
TABLE 18.2(c) Natural/Semi-synthetic Media.

C. Hagem's Malt Agar (modified after Modess 1941).

Malt Extract (Boots Pure Drug Co.)	5g.
Glucose	5g.
KH_2PO_4	0.5g.
$MgSO_4.7H_2O$	0.5g.
NH_4Cl.	0.5g.
$FeCl_3.6H_2O$ (1% soln).	5mg.
Distilled Water	to 1000 ml.
Oxoid Agar No.3	15g.

P L A T E 29.

ILLUMINATED COOLED CABINETS.



ILLUMINATED COOLED CABINETS (FIGURES 16.0 A, B & C)

KEY

LIGHTING HOOD

A. Rocker switches and neons

B. 4 4′ 'daylight' fluorescent tubes

C. Capacitors and ballast

D. Sealed clear perspex partition

MAIN CABINET. EXTERNAL FEATURES

E. Foam-rubber seal

F. 'Humex' thermostat and temperature sensor

G. Air inlet and outlet

H. Sealed sliding inner perspex door

I. Cabinet door (push-fit)

J. Rivited right angle aluminium frame

MAIN CABINET. INTERNAL FEATURES

K. Plastic tubing serving as air-ducting

L. Polypropylene-glassfibre ('Celmar')/Expanded polyurethane/Epoxy-resin Laminate

M. Polypropylene welded seams

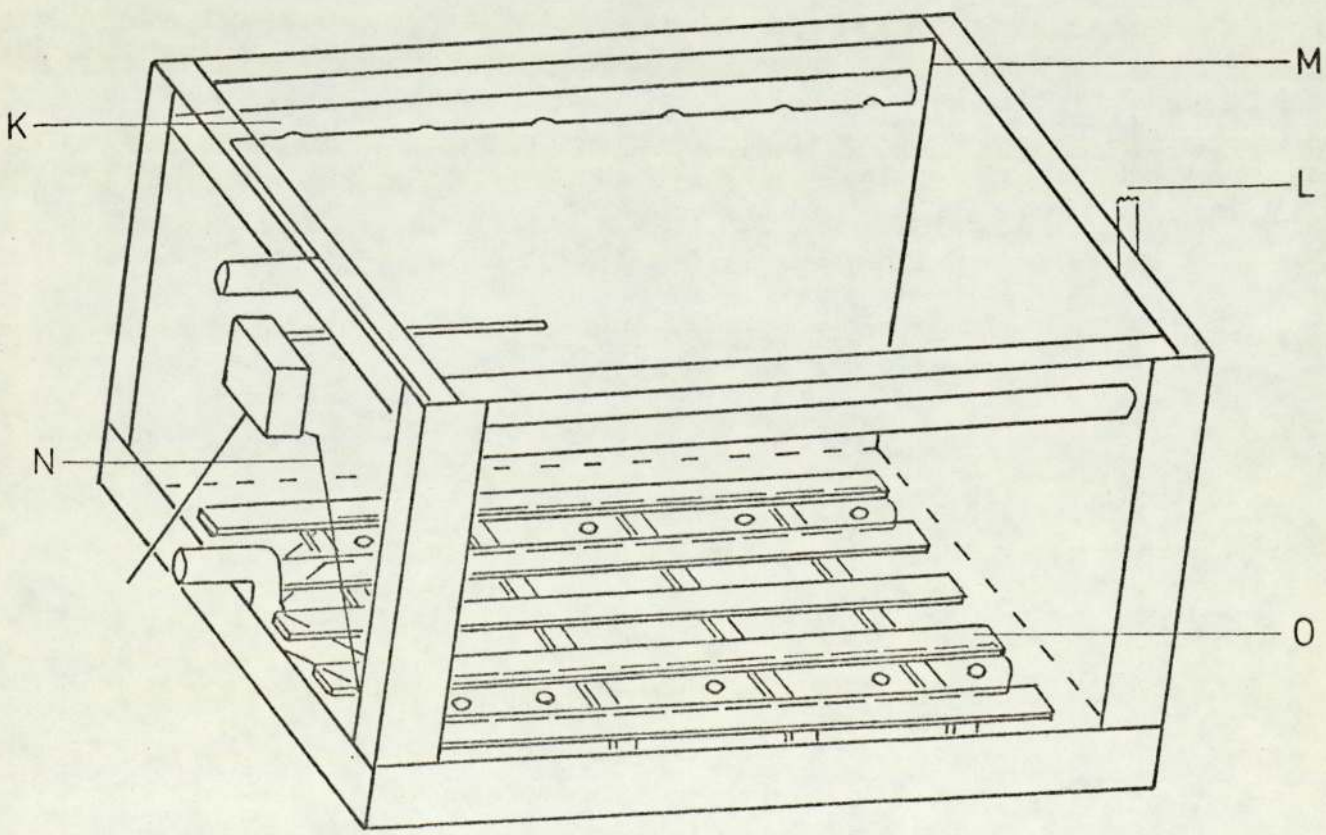
N. 'Humex' air-warming cable fitted beneath wooden support rack

O. Wooden support rack

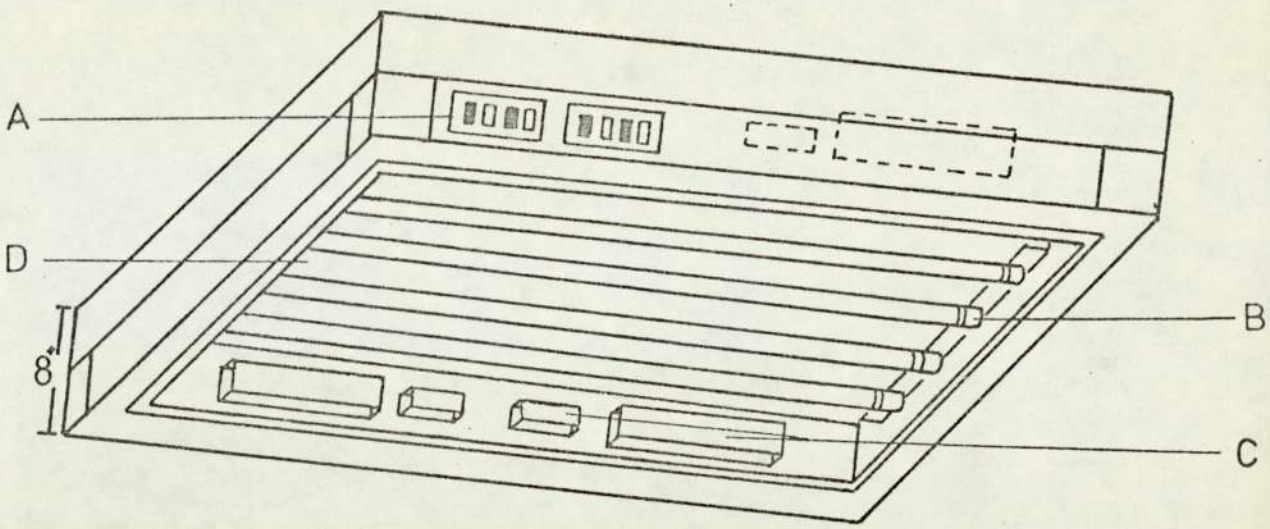
CONSTRUCTION & OPERATION DETAILS

- Overleaf

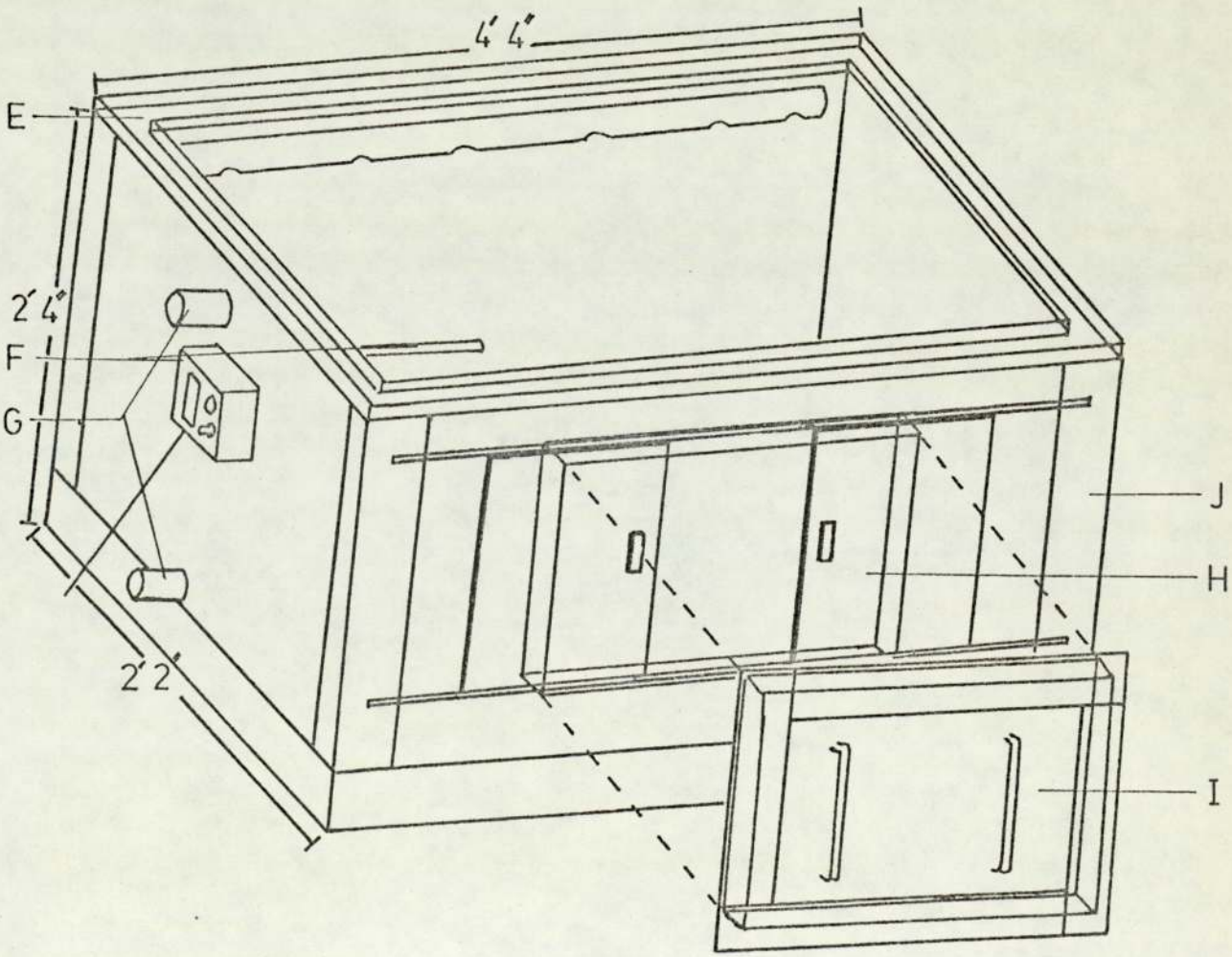
C. MAIN CABINET. INTERNAL FEATURES



A. LIGHTING HOOD



B. MAIN CABINET. EXTERNAL FEATURES



ILLUMINATED COOLED CABINETS.

Construction and Operation Details.

The cabinets (internal dimensions: 4' x 1'10" x 2'2"; with 4' x 1'10" x 2' headroom) were constructed from a 2" width insulating plastic laminate (Heat Transfer Coefficient 0.044) (D.P.Dawnpress Ltd. Stockport, Cheshire). The top section consisted of a single unit lighting hood (internal dimensions: 4' x 1'10" x 6") which was seated on a foam rubber flange (mounted on the upper rim of the main cabinet) and sealed from the main cabinet by a detachable sheet of clear perspex.

Static incubation within the main cabinet was provided by means of a 'Humex' air-warming system, the control box and temperature sensor rod were mounted on the wall of the cabinet, the air-warming cable passed beneath the bottom wooden support rack and was clipped to its undersurface in a series of longitudinally running loops.

Air circulation was maintained through 2 branched piping systems (c. 2" dia., black plastic pipes perforated by a series of c.$\frac{3}{4}$" dia. holes - pipes from Plastic Constructions Ltd., Birmingham), one below the support rack, the other attached to the wall sections immediately beneath the lighting hood; the twin pipes of both sets passing the length of the cabinets.

The lighting hood contained 4 individually wired 4' 'daylight' fluorescent lights with separate capacitors and ballast linked to externally positioned rocker switches and neons. These formed individual 'instant start' self-starting units (electrical equipment obtained from Walsall Conduits, Aston, Birmingham). The hood size was designed to take a range of cinamoid filters (to investigate possible action spectra for fruit-body formation) clipped or taped to the base

of the perspex sheet . (Coloured filters from Strand Filters, London).

The cabinet was able to accommodate 18 - 6" square by 7" deep white plastic growing containers.

The wall sections consisted of a bonded sandwich of polypropylene sheet (backed with fibreglass reinforcing net - marketed by British Celanese Ltd., London), which provided smooth, clean and chemically resistant internal surfaces and further enabled the wall sections to be sealed internally by the use of polypropylene welding techniques (materials supplied by Plastic Constructions Ltd., Birmingham) forming watertight seams. In fabrication of the insulated wall section by the manufacturers (D.P.Dawnpress Ltd.), the polypropylene/fibreglass sheet is epoxy-resin bonded to expanded polyurethane blocks which are supported by an internal framework of fibreglass uprights; the external surface is finished by a layer of fibreglass / epoxy - resin and coated with a smooth surface white gloss.

Externally the cabinets were supported by a framework of aluminium right-angle (2" x 4") lengths drilled and attached through the outer wall only, by means of specially designed expanding rivets (Linreed Ltd., Birmingham), which formed a 3 pronged anchor attachment at the outer polyurethane/epoxy-resin interface. This arrangement effectively caused minimal interference with the insulating properties of the plastic laminate and produced uninterrupted internal surfaces.

Access was gained by an external 'push-fit' insulated door and an internal sliding clear-perspex observation door forming an internal seal.

Atemperation.

Air over a range of temperatures, (10-50°C) was supplied to the cabinets from a single specially constructed air-conditioning unit. (Lec Refrigeration Ltd.). Provision was made in constructing the

cabinets for recycling air through the blower/conditioner unit in order to attain and maintain low temperatures.

Further Modifications.

The cabinets were prototypes and provided a useful though relatively unsophisticated lighting plus cold temperature facility. However, further modifications required to be introduced to provide (a) improved air distribution; (b) humidity control linked to operation of the conditioner unit (particularly at low temperatures where icing-up occurs); and (c) cooling for the lighting-hood with the maintenance of certain lighting conditions and cabinet temperatures.

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PHYSIOLOGICAL STUDIES OF GROWTH AND DEVELOPMENT IN THE THREE BASIDIOMYCETE SPECIES *LEPISTA NUDA* (BULL. ex FR.) COOKE, *LEPISTA SAEVA* (FR.) ORTON AND *CALOCYBE GAMBOSA* (FR.) SINGER.

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Thesis submitted in fulfilment of the requirements of the degree of Doctor of Philosophy. November 1976

ABSTRACT

Studies were undertaken into the general physiology and fruiting of three wild Basidiomycetes, Lepista nuda (Bull. ex Fr.) Cooke, (wood blewit), Lepista saeva (Fr.) Orton, (pasture blewit) and Calocybe gambosa (Fr.) Singer, (St George's mushroom). The three species were formally grouped together within the genus Tricholoma on overall fruit-body characteristics, but have since been subdivided on the basis of detailed ultrastructure of the fruit-body and spores into the genera Lepista and Calocybe both within the family Tricholomataceae.

C.gambosa is a vernal species, both Lepista species produce fruiting bodies in late autumn. L.nuda occupies a variety of habitats in particular accumulations of partly decomposed leaf litter and rich soils, fruiting in troops under sheltered conditions. L.saeva and C.gambosa generally occupy more open positions in pastures, meadows and fringing woods, often forming 'fairy-rings', and occasionally within woods.

Results from screening a large number of isolates within each species for P-diphenol oxidase and cellulase activity indicate considerable diversity within species and between species. Response to a range of temperature and hydrogen ion concentrations for a smaller number of isolates indicate a more uniform response within the individual species range.

Growth of a restricted number of isolates within each species was determined on a large number of individual carbon and nitrogen sources at a selected carbon:nitrogen ratio. Vitamin, trace metal and fatty acid nutrition was examined for selected strains. Good growth and a wide nutritional diversity was shown by isolates of L.nuda and L.saeva on a wide range of mono-, di-, tri- and polysaccharides, sugar alcohols, hemi-cellulose, cellulose and many nitrogen sources. C.gambosa generally showed restricted growth except where the basal medium was supplemented with natural extracts.

The effect of a range of oxygen and carbon dioxide concentrations was assessed for individual species.

A number of L.nuda and L.saeva isolates were selected for fruiting trials on solid substrates based on their performance in specially constructed tubes and in flasks. Three strains of L.nuda used throughout the survey fruited successfully under artificial conditions, one strain of which almost completed fruit-body maturation on a natural agar medium under simulated enviromental conditions.

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

L I T E R A T U R E R E V I E W

C O N C L U S I O N S

LITERATURE REVIEW

1:0 INTRODUCTION

A review of the literature concerning Lepista nuda , Lepista saeva and Calocybe gambosa reveals that most information available deals with various aspects of the physiology of L. nuda with a relative paucity of information concerning L. saeva and C. gambosa.

The literature from which the information is largely taken deals with comparative studies within the genus Tricholoma (to which these species formally belonged) such as those of Norkrans (1950, 1953) and Rawald (1962 a & b, 1963) and to physiological investigations within fairly heterogeneous groupings of fungi considered to be ectomycorrhizal on certain forest tree species; these include the studies of Lamb (1974) and Lundeburg (1970). L. nuda has also stimulated interest as an edible, rapidly growing and nutritionally unexacting fungus for submerged culture studies and for the production of fruitbodies, it has arroused interest as a fungus which is fairly resistant to contaminants and which produces a wide range of antibiotics (Melik-khachatryan, Abramyan & Gasparyan (1970)).

L. saeva is less frequently encountered and C. gambosa a comparatively difficult fungus to culture,have both received far less attention; this may also be reflected in the fact that neither has been considered to be a mycorrhiza former of any consequence.

The literature as indicated is diverse and neither of these species has singly received detailed attention except L. nuda for a submerged culture study and some pioneering work on fruitbody production by Constantin and Matruchot. The results of individual investigations have been reported factually from data of the individual authors, however, a number are open to serious criticism which is detailed in the following section.

Low yields were obtained by Norkrans (1950, 1953) for L. nuda isolates, dry weights rarely exceeding 15 mg for a 30 day incubation, the best yields with amino-acid or organic acid supplemented

media being about 35 mg following a 35 day growth period, poor yields were also recorded by Modess (1941). Lundeberg (1970) also achieved low yields for his L. nuda isolate of only approx., 60 mg dry weight following 50 days growth on a heterogeneous natural medium in stationary liquid culture.

Very poor growth for C. gambosa isolates is also given by Norkrans (1950, 1953) with yields generally not exceeding 6 mg with 35 days incubation, often falling below 4 mg. The best growth for amino-acid or organic acid supplemented media, for the fastest growing of the three isolates employed in the combined studies, was 47 mg after 60 days incubation.

Rawald (1962 a & b, 1963) relates yields to control values rated at 100 for growth response to a variety of carbon and nitrogen sources and vitamin additions. The controls lacked the substance under study, however, yields generally not exceeding the control value by 200 to 300 per cent, exceptionally 500 per cent for certain of the nitrogen sources, would indicate exceptionally high yields for the controls or overall very low yields for treatments.

The exceptionally low yields shown or indicated by these investigators must cause results for certain of the sections to be treated with reserve as they indicate either the unfortunate choice of extremely slow growing isolates or the use of a medium which is markedly unfavourable to growth. In either event, such phenomena cause a serious detraction from the validity of the results.

Improved yields were given by Lamb (1974) for his isolate of L. nuda on a variety of carbon sources in a synthetic medium containing ammonium chloride as nitrogen source. Many yields were in the 80-125 mg dry weight grouping following 42 days incubation, startling increases in growth of 615, 819 and 1130 per cent were obtained on dextrin, maltose and inulin, as carbon sources when the medium was supplemented with 0·1g/L addition of 'start glucose'.

Votýpka (1972) records much improved growth for L. nuda with rates of 3.5 mm growth per day achieved over malt agar plates.

Very good yields for growth of L. nuda isolates have been reported for submerged culture studies. Reusser, Spencer and Sallans (1958a), Falange, Smith and Rackis (1964) and Falange (1962) record isolates of L. nuda as growing better or equally best among a range of isolates screened for growth on a variety of media. Voltz (1972) in shake flask culture achieved yields between 300 and 418 mg dry weight for the best carbon and nitrogen sources utilised over the 14 day growth period.

Growth on semi-solid media and in stationary liquid culture is probably a better reflection of the adaptive capacity of a fungus in nature, than the growth of strains resulting from the strongly selective pressures encountered under conditions of submerged culture.

The reports concerning fruitbody production by L. nuda were most detailed as performed by Constantin and Matruchot in 1898. Both their attempts, however, and those of the others cited, amount to little more than a transferal of material outside to more sheltered conditions protected from extremes of temperature and desiccation, these studies combined provide almost no information on important facets of culture on solid substrates such as, inoculum potential, growth rate, substrate preference, the factors required for fruitbody formation and the detailed aspects of fruitbody development.

The species names used throughtout the literature survey are those adapted by the individual authors. The nomenclature of these species is dealt with in detail in a later section.

2:0 EFFECT OF PHYSICAL PARAMETERS ON GROWTH.

2:1 Temperature

Norkrans (1950) determined temperature profiles on

glucose-ammonium tartrate minimal medium supplemented with yeast extract over the range 17.5 to 30°C. Temperature optima recorded for T. nudum I and T. gambosum II were 25°C and 20°C at 20, 30 and 30 and 60 days respectively. Growth was prevented at 27.5°C for T. nudum and reduced for T. gambosum. Zadražil (1973) reports temperature optima between 15-20°C for an isolate of L. nuda grown on malt agar.

2:2 Hydrogen Ion Concentration.

Norkrans (1950) reports pH optima for 2 strains each of T. nudum and T. gambosum on a glucose-asparagine nutrient solution, strain II on media where ferric citrate replaced ferric chloride as the iron source. Optima recorded were pH 6.0 at 25 and 20 days for T. nudum strains I and II respectively and pH 5-6.0 and 6.0 for isolates I and II of T. gambosum at 30 days. No shift in pH over the incubation period was recorded, however, on a glucose-ammonium tartrate medium under identical conditions of phosphate buffering where thiamine addition was replaced by 50 ppm of pyrimidine a pH drop of 6.0 to 4.1 and 3.5 was recorded following 30 and 50 days incubation of T. nudum II.
Lundeberg (1970) reports a pH shift during incubation of L. nuda to lower values.

A shift in the hydrogen ion concentration during growth is largely a function of the nitrogen source, an unusually strong pH displacement was recorded by Rawald (1963) where ammonium salts of strong acids were employed for culture of T. georgii and T. nudum isolates.

In submerged culture studies with T. nudum somewhat lower pH optima are recorded, Reusser, Spencer and Sallans (1958b) report optima at pH 3.0 - 5.0 and Voltz (1966) with pH 7.2 and 4.5 at 10 and 20 days incubation respectively.

3:0 UTILISATION OF CARBON SOURCES.

Lamb (1974) conducted a thorough study on the growth of a

range of Basidiomycetes on single carbon sources in a synthetic medium containing ammonium chloride as sole nitrogen source. Best growth was obtained with an isolate of L. nuda on D-glucose, D-mannose, cellobiose and pectin, and moderate development on galactose, mannitol, glycogen and starch. Poor utilisation was achieved with maltose, sucrose, lactose, dextrin and raffinose, however, addition of 0.1 g/L of D-glucose to the medium in the form of 'start glucose' caused fructose, D-galactose, maltose, starch, inulin and glycogen to respond markedly as improved sources of carbon. (Start glucose is a small quantity of added glucose, insufficient to significantly affect yield but sufficient to provide the energy for initial growth and the induction of enzymes for metabolism of sugars that cannot readily be assimilated by the organism). Generally the hexitol, L-rhamnose and the sugar alcohols erthritol, inositol, sorbitol and the corresponding monosaccharids D-erythrose, L-sorbose and D-arabinose permitted little development.

Rawald (1962b) for L. nuda gives best yields for fructose and xylose. Dry-weights obtained for D-galactose, D-glucose, and sucrose were good, growth was poor on D-arabinose and comparable to a carbon-free control on cellulose and pectin. Yields obtained by Rawald for an isolate of L. personata are best for D-xylose, D-galactose, D-glucose, and fructose, moderate growth was obtained with sucrose and starch, poor development on lactose, cellulose and pectin. Empirical comparisons were made for polysaccharides on solidified media using an index of mycelial extension and density, yields for mono-and disaccharides were recorded as dry-weights following growth on a synthetic medium containing ammonium carbonate as sole nitrogen source.

Wilson (1970) recorded growth of T. personatum on cellobiose, glucose and maltose solidified media and demonstrated typical highly branched 'colonial' morphology on cellobiose and similar growth on glucose and maltose; the carbohydrate source was demonstrated to influence activity of cell wall hydrolysing enzymes and hyphal branching.

Norkrans (1950) gives best growth for T nudum on starch,

sucrose, maltose, lactose and fructose respectively. Xylose, raffinose, mannose, and inulin also enabled good growth, poor development only being obtained with galactose. Addition of 'start glucose' (0.5 g/L) to starch and inulin did not significantly increase yields.

Reusser, Spencer and Sallans (1958 a) selected T. nudum from among 10 Ascomycetes and Basidiomycetes grown in submerged culture on synthetic, molasses and waste sulphite media (effluent from the sulphite process in paper pulp manufacture), on the basis of high yields and high protein content; grown in stationary cultures on a synthetic ammonium sulphite medium the T. nudum isolate utilised 25 sources of carbon listed in order of decreasing yield of dry-matter; pyruvate, xylan, sucrose, maltose, D(+) mannose, raffinose, D-arabitol, L(+)-rhamnose, D-galactose, D-fructose, D(+)xylose, dextrin, glucose, D-ribose, cellobiose, sorbitol, L(+)arabinose, starch (soluble), dulcitol, mannitol, inulin, salicin, lactose, L(-)sorbose and cellulose.

Reusser, Gorin and Spencer (1960) showed that T. nudum grown on sucrose (4%), molasses and raffinose media, preferentially utilised the glucose leaving fructose in the fermentation broth. The fructose could be purified and recovered. They suggested that glucose may inhibit fructose assimilation. Good growth was also recorded on galactose and melibiose.

Worgan (1968) reports obtaining good growth of T. nudum on a medium containing xylose as sole source of carbon.

In shake-flask culture on the medium of Humfeld and Sugihara (1952), Voltz (1972) reports growth of an isolate of L. nuda on a large number of mono-, di-, and polysaccharides also including a range of organic acids and miscellaneous carbon sources. Outstanding dry-weights were obtained with D-mannose and cellobiose, good yields with D-glucose, D-fructose 1,6 diphosphate and pectinol-A. D-fructose, xylan, pectin and galactouronic acid gave moderate growth, poorer development being obtained with DL-arabinose, maltose and raffinose. L-sorbose, and α & β lactose,

sucrose, glycogen, starch, tannic acid, i-inositol, D-mannitol, D-sorbitol and casein gave poor yields. Dry-weights similar to the carbon free control value were obtained with D-arabinose, D-ribose, D-xylose, citric acid, DL-malic acid, oxalic acid, pyrogallic acid, resorcyclic acid, succinic acid, D-tartaric acid and D-glucosamine hydrochloride.

3:1 Growth on Cellulose and Lignin.

Norkrans (1950) records growth on and clearing of opaque cellulose agar in Petri-plates by isolates of T. nudum and T.gambosum.

Lindeberg (1946) and Norkrans (1950) cultured isolates of T.gambosum and T.nudum plus T.personatum respectively on sterilised Glyceria straw in flasks for periods of 165 days and 8 months. T.gambosum decomposed 16.1% of the dry-matter and 18.7 and 24.2% of the lignin and cellulose content. T. nudum removed approximately 37% of the dry-matter and decomposed about 23 and 65% of the lignin and cellulose. T.personatum decomposed approximately 30% of the dry-matter and about 3 and 66% of the lignin and cellulose. T. personatum only demonstrated a capacity to attack lignin where the flask contents were supplemented with either 25 or 125 mg glucose. However, Norkrans reports a positive Bavendamm test for this species.

In in vitro studies on cellulolytic enzyme preparations from T. nudum, Norkrans (1950) demonstrated an optimum temperature between 30 and 37°C and considerable heat stability - total inactivation occurring only at 100°C. The enzyme preparation exhibited a pH optimum in the range of 4.5 to 5.0 and maximum stability over the range 4.0 to 6.0, however, it was not readily inactivated by pH alteration over the range 1.2 to 10.0 prior to readjustment to pH 5.0 Cellobiose at 0.03% caused in excess of 50% inhibition of cellulolytic ability, total replacement of cellulose with cellobiose permitted cellulolytic activity amounting to 20-30% of that of the pure cellulose control value. This repression was also shown by glucose and a number of sugars.

Growth on lichenin (lichenin prepared from Icelandic moss,

Cetraria islandica is 73% glucose 1,4 linked and 27% in a 1,3 position; this produces an irregular chain giving lichenin an open easily attacked structure) and cellulose, the latter with and without 'start glucose' addition, was investigated for T. nudum and T. gambosum by Norkrans (1950). In both cases lichenin was more readily attacked and served as a better carbohydrate source. 'Start glucose' did not assist utilisation of the cellulose for either species.

Norkrans (1950) records being unable to show cellobiose, mannan, starch or maltose splitting enzymes in crude enzyme preparations from T.nudum following incubation periods of varying lengths. However, enzymes attacking native and regenerated cellulose, salicin, sucrose and raffinose were indicated by the presence of reducing breakdown products.

Lundeberg (1970) reports total inability of his isolate of T. nudum to produce cellulase, together with trace pectinase following 50 days incubation, but strong proteinase and laccase activity on a medium containing tannin, cellulose, pectin, malt extract and bone meal.

T. nudum was shown to grow well in submerged culture on waste sulphite liquor, a waste product resulting from the removal of lignin in paper-pulp manufacture (Reusser, Spencer and Sallans 1958 a & b), however, they report that it was unable to utilise the lignin hydrolysates vanillin, syringealdehyde and p-hydroxybenzaldehyde when grown in stationary culture.

4:0 UTILISATION OF NITROGEN SOURCES.

Norkrans (1950) reports yields of T. nudum I on a glucose-salts basal medium as best with asparagine, urea and ammonium tartrate. Good growth was recorded on glycine, alanine and ammonium hydrogen phosphate. Moderate yields were obtained on potassium nitrate, ammonium sulphate and ammonium chloride. Leucine gave poor development.

Very poor growth was obtained with T. gambosum I with extended

incubation under the same conditions. Yields did not exceed 3.3 mg. Potassium nitrate did not appear to be utilised, otherwise the figures were not significantly different over the range of nitrogen sources listed for T. nudum I.

Lundeberg (1970) reports best yields of T. nudum obtained on asparagine, with moderate yields on ammonium tartrate and glycine. Yields were not obtained in excess of the nitrogen-free control value with potassium nitrate, acetamide, diethylamine, D-glucosamine, L-proline or pyridine. Potassium nitrite totally inhibited growth.

Rawald (1963) records the best yield for T. nudum on propionamide; good growth was obtained relative to the control with asparagine, glycine, ammonium tartrate, urea and peptone, these values were just superior to those obtained on casein hydrolysate and leucine. Average yields were given on triethylamine, sodium nitrate, potassium nitrate, and valine. Poor yields were produced with valeric acid amide, sodium cyanide and sodium nitrite. Ethyl cyanide, ammonium carbonate and acetonitril gave yields only slightly better than the nitrogen free control. Ammonium chloride gave yields equivalent to the control value, sodium rhodanid proved inhibitory to growth.

Lack of growth obtained by Lundeberg (1970) on acetamide contrasts with the excellent yields obtained with propionamide indicated by Rawald for T nudum. Growth of T. nudum, though small, on sodium nitrite was unusual.

Dry-weights obtained with C.georgii by Rawald were superior with peptone, good yields were given on asparagine, propionamide and ammonium tartrate in relation to the control value. Casein hydrolysate was an improvement on acetonitril, leucine and betaine hydrochloride, which are indicated as better than average sources of nitrogen for this species. Sodium cyanide, triethylamine and collectively urea, ethylcyanide and potassium nitrate were respectively moderate yielding nitrogen sources. Valeric acid amide, ammonium chloride and ammonium carbonate produced values

falling to and similar with that of the nitrogen free control. Sodium rhodanid and sodium nitrite caused inhibition of growth.

Voltz (1972) records yields obtained from growth on amino-acids and related nitrogen sources substituted into Humfield's and Sugihara's medium (1952) for L. nuda grown in shake-flask culture.

Glycine produced yields superior to all nitrogen sources tested. Peptone and DL-alanine at approximately half this value yielded well, L-glutamic acid, DL-methionine and aspartic acid produced dry-weights, at about a quarter of that obtained with glycine, were fair nitrogen sources. L-Asparagine and DL-valine gave low yields. L-proline, creatine, betaine hydrochloride only slightly bettered the nitrogen-free control value which equalled yields on DL-isoleucine, DL-threonine & DL-tryptophane . L-cystine, D-histidine and cysteine hydrochloride caused growth inhibition.

In comparison with the ammonium and nitrate nitrogen sources tested, ammonium bicarbonate and urea yielded somewhat higher than peptone, ammonium tartrate supported poor growth similar to L-asparagine. Magnesium nitrate and ammonium nitrate gave slightly improved yields to that of the nitrogen-free control. Values almost identical to that of the control were given on sodium nitrate, potassium nitrate, ammonium sulphate and ammonium chloride. Inhibition of growth was encountered with cobaltous ammonium sulphate, ammonium citrate, cupric nitrate, cobaltous nitrate and uranium nitrate as nitrogen sources.

Reusser, Spencer and Sallans (1958 b) list nitrogen sources utilised for a T. nudum isolate on a synthetic glucose-salts medium in stationary cultures. Double the quantity of nitrogen was added for racemic mixtures where it was assumed only the L-isomers were utilised. Organic nitrogen sources utilised were : L-asparagine, DL-alanine, glycine, DL-serine, L-arginine, L-proline, C-threonine, L-glutamine, DL-valine, L-tyrosine, L-leucine, DL-leucine, DL-isoleucine, DL-alanine, L-histidine, DL-lysine L-tryptophane, DL-∝-aminobutyric acid, DL-glutamic acid, DL-methionine, DL-aspartic acid and DL-phenylalanine, L-taurine was not utilised.

Inorganic nitrogen sources utilised were potassium nitrate, ammonium nitrate, ammonium tartrate, urea, ammonium phosphate (dibasic) ammonium phosphate (monobasic), ammonium chloride and ammonium sulphate.

Varying the ammonium tartrate concentration of the standard glucose-ammonium tartrate basal medium, between 0.025 and 1 per cent was found to increase crude protein levels from 18 to approximately 50 per cent and decrease fat from 24 to 4 per cent.

Reusser, Spencer and Sallans (1958 b) and Rawald (1963) both note poor growth where the ammonium salt of a strong acid was used, which resulted in a rapid fall in pH to inhibitory levels. Reusser et al., (1958 b) found that adopting a mediumof high buffering capacity enabled satisfactory yields to be obtained on compounds such as ammonium sulphate.

5:0 VITAMIN REQUIREMENT FOR GROWTH.

Norkrans (1950) for T. nudum II on a glucose-ammonium tartrate basal medium records strongest development on yeast extract, only approximately two-thirds of this extent of growth was attained on a synthetic yeast-extract vitamin 'M'. (Vitamin mixture 'M' was composed of biotin, thiamine, calcium pantothenate, "folic acid", nicotinamide, p-aminobenzoic acid, pyridoxine, riboflavin, choline and inositol). Growth with aneurin, aneurin-biotin, aneurin-inositol and aneurin-biotin-inositol produced similar yields at less than half that attained on yeast extract and a third below that produced with the synthetic vitamin mix.

T. nudum I did not show significant stimulation of growth on vitamin-mixture 'M' compared to growth on aneurin or a combination of aneurin, biotin and inositol. Removal of calcium pantothenate from the synthetic vitamin mix caused a reduction in yield to slightly more than half that obtained with 'M'. Removal of choline, "folic acid" nicotinamide or riboflavin singly from the mixture 'M' did not cause any discernable alteration in yield, subtraction of p-aminobenzoic acid and pyridoxine caused a slight elevation in yield relative to 'M'.

Vitamin mixture 'M' almost doubled the dry-weights obtained with T. gambosum with aneurin or collectively aneurin, biotin and inositol. Calcium pantothenate caused a drop in yield to a value in slight excess of that obtained with aneurin. Exclusion of pyridoxine, p-aminobenzoic acid and nicotinamide from the vitamin mix slightly increased yield/ compared to the complete mix. Exclusion of choline, "folic acid" and riboflavin did not significantly alter yield.

Growth of T. nudum I and T. gambosum I isolates on the basal medium only took place where intact aneurin, or the pyrimidine moity of the two thiazole and pyrimidine moities comprising aneurin, was present.

In an investigation of the effects of suboptimal amounts of pyrimidine on growth of T. nudum I and II and T. gambosum II isolates, a direct proportionality was shown in the amount of growth obtained and the corresponding quantity of the pyrimidine moity supplied over the range 0, 25, 50, 100 µµmol pyrimidine. The addition of 4 m µmol of pyrimidine following cessation of growth in the 50 µµmol series caused an immediate resumption of strong growth.

From maximum yields of T. nudum isolates I & II in the 50 µµmol series the mycelium: pyrimidine quotient (M/P) was calculated as 1.0 and 1.8×10^6 respectively or on a molecular basis 2.2 and 3.7×10^8 respectively.

Kögl and Fries (1937) for an isolate of T. nudum obtained almost absence of growth in the vitamin free control and with the addition of biotin and inositol. Aneurin and biotin, inositol and aneurin produced growth to a comparible extent.

Rawald (1962) studied the vitamin requirements of C. georgii, L. personata and several strains of L. nuda utilising a glucose-ammonium carbonate basal medium.

L. nuda produced best yields supplemented with pressed

brewers and bakers yeasts respectively, which stimulated growth considerably more than biotin, which was individually superior to thiamine, calcium pantothenate and p-aminobenzoic acid. Pyridoxine hydrochloride and niacinamide produced just significantly more growth than the vitamin-free control.

For six strains of L. nuda addition of vitamins individually produced just significantly greater growth than that obtained for the control with thiamine (in 2 strains) and biotin, pyridoxine hydrochloride and inositol (in 1 strain each).

Growth of L. personata was best with thiamine, yields declined to the control value respectively with pressed brewers yeast, pressed bakers yeast and calcium pantothenate followed by a mixture of vitamins in the B complex then inositol and finally biotin which was significant with respect to the vitamin -free control value.

C. georgii showed greatest growth on thiamine addition, growth in excess of, but declining to that of the control value was obtained with pressed bakers yeast followed collectively by pressed brewers yeast and calcium pantothenate.

Reusser, Spencer and Sallans (1958 b) for their work with T. nudum on synthetic glucose-ammonium sulphate medium employed thiamine, pyridoxine and p-aminobenzoic acid as vitamin additions.

6:0 EFFECT OF AMINO-ACIDS, AMIDES AND ORGANIC ACIDS AS GROWTH FACTORS.

Norkrans (1950) investigated the effect of casein hydrolysate supplement (25 mg/L) to the glucose-ammonium tartrate basal medium also the effect of 21 different amino-acids added singly inthe proportion that they occured in the casein hydrolysate preparation calculated on the basis of 25 mg. With T. gambosum growth was most strongly promoted with glutamic acid, hydroxyproline and histidine. Tyrosine and threonine markedly inhibited growth. Casein hydrolysate did not accelerate the growth rate of T. nudum, tryptophane, valine and arginine caused an increase in dry-weight above

that of the control. Tyrosine, histidine and isoleucine caused inhibition of growth, the remaining amino-acids had hardly any effect. In neither case did the level of growth promotion indicate a requirement for essential amino-acids by these isolates.

Norkrans (1953) examined the effect of aspartic and glutamic acids and their corresponding keto-acids and half amides as growth stimulating substances for isolates of T. nudum and T. gambosum III especially during the initial stages of growth. Substances were added at a concentration of 10 μ mols per flask (0.4 m mols/L) to either a glucose-ammonium tartrate or glucose--ammonium hydrogen phosphate synthetic medium. Asparagine and glutamine produced the greatest response with T. gambosum giving relative growth values of 525 and 375 compared to a control of 100 after 30 days. Oxaloglutarate proved to double dry-weight, whereas its amino-acid glutamic acid had no effect.

Casein hydrolysate at 0.184g/L was more beneficial than asparagine in its effect over the 60 day incubation period. The dipeptide glycyl-glycine promoted tripled mycelial production of T. gambosum whereas singly glycine exerted only slight effect. This finding, together with the previously observed stimulative effect of asparagine and glutamine, is postulated by Norkrans to reflect certain difficulties in peptide synthesis by this species.

In a separate experiment with T. gambosum where additions of 250, 50, 10 and 2 μmols of the respective substances were used, maximum growth effect was obtained with 10 μmol additions in the aspartic acid series but 50 or 250 μmol in the glutamic acid series.

These substances produced only a slight growth promotive effect on the T. nudum isolate, values not in excess of 18 mg were achieved, the keto-acids appearing to be more stimulatory than either of their corresponding amino-acids or amides.

6:1 Leaf litter extracts as sources of Growth Factors.

Norkrans (1944) recorded that aspen-litter extract at low concentrations exercised a growth-promoting influence on several Tricholoma species, however, this effect was reversed at higher concentrations with growth inhibition apparent.

Melin (1944) reported leaf-litter extracts of maple, beech, oak, aspen and pine stimulated litter-decomposing hymenomycetes to a particularly high degree. The litter decomposers were stated to be insensitive to water soluble inhibitory substances contained in the litter which inhibited or prevented growth of many soil hymenomycetes, particularly mycorrhizal fungi.

7:0 INFLUENCE OF LEAF-LITTER ASH AND METAL IONS.

Norkrans (1950) did not report growth stimulation of either T. nudum or T. gambosum isolates with zinc or manganese addition to a basal medium containing potassium dihydrogen orthophosphate, magnesium sulphate, sodium and ferric chloride as inorganic constituents.

Addition of calcium at 9 mg/L caused an increase in yield, added at four times this amount growth equalled that at this higher level of calcium with zinc and manganese additions and also with aspen foliage litter plus this metal ion combination.

The effect of ash alone, also the higher level of calcium, were particularly stimulatory to the growth of T. nudum. The growth promotory effect of the ash addition was considered to be largely due to its calcium component, an element which is well documented for its ability to nullify the inhibitory action of excessive levels of other metal ions in the culture medium.

8:0 FATTY ACID COMPOSITION AND ASPECTS OF BIOSYNTHESIS.

Following observations on variation in fat content of T.nudum

mycelium by Reusser, Spencer and Sallans (1958 b) resulting from alteration to the nitrogen level of the glucose-ammonium tartrate medium employed for submerged culture studies (levels of 4.2 to 24.4 per cent fat at C:N ratios of 16 and 384:1 respectively), several detailed studies were instigated into the composition and mode of synthesis of fats by T. nudum in a low nitrogen medium.

Leegwater, Youngs, Spencer and Craig (1962) investigated the fatty acid composition of the mycelium by thin layer, column and gas-liquid chromatography in conjunction with a number of additional novel techniques.

At two days incubation, while still in the lag phase of growth, this revealed the neutral lipid fraction (the larger component) to contain as the major fatty acids palmitic, oleic and linoleic acid; stearic acid constituted a lower proportion. Myristic, palmitoleic and linolenic acids were represented in small amounts.

Linoleic acid was the major fatty acid of the phospholipids with palmitic, stearic and oleic as minor components, myristic, palmitoleic and linolenic were present in a small quantity.

By contrast in 1-day-old cultures the composition of both fractions was distinctly different, with linoleic acid the main fatty acid, linolenic acid being also present as a major component of the triglycerides during this period, indicating that linoleic acid, and its closely related fatty acid, linolenic acid play a special role in the young developing mycelium.

Analysis of the major components at 4 days incubation revealed the neutral lipids to consist largely of triglycerides with trace quantities of ergosterolesters, free fatty acids, a higher level of ergosterol plus a number of unidentified non-saponifiable compounds.

The phosphlipids were above half phosphadyl-choline in

composition and approximately a quarter phosphadyl ethanolamine. Phosphatidyl serine and phosphatidic acid plus an inositol containing phospholipid were present in minor amounts.

In radioactive tracer studies utilising glucose-U-C^{14} Leegwater and Craig (1962) showed T. nudum mycelium incorporated label into the linoleic fraction of the triglycerides after a considerable lag compared to that of palmitic, stearic and oleic fractions. Incorporation of linoleic acid into the phospholipids was in the same proportion to that of the other major fatty acids, this led to them postulating possibly separate fatty acid pools for neutral and phospholipid synthesis. Although a pathway of oleic acid conversion to linoleic acid was established, they concluded that this anomalous labelling pattern of linoleic acid indicated the involvement of, as yet, unidentified pathways, also, that the rate and order of fatty acid synthesis is largely dependant on experimental conditions.

8:1 Growth Response to Aliphatic Alcohols and Fatty Acids.

The importance of fatty acids and alcohols in the nutrition of isolates of L.nuda, L.saeva and C.gambosa, has not received consideration though has proved of considerable significance in the physiology of certain fungi. Information concerning those basidiomycete species which have elicited a marked growth response is summarised in this section.

Wardle and Schisler (1969) for single-spore isolates of the cultivated mushroom, Agaricus bisporus reported growth stimulation of between 4 to 6 times that obtained on the xylose-asparagine basal medium following the addition of a range of animal and vegetable oil and fat supplements, best growth was obtained with cottonseed and corn oils.

Ethyl and methyl esters of oleic and linoleic acid added at levels similar to that found in cottonseed and corn oils produced a growth response to a similar extent. The mycelial growth response did not show a linear relationship, suggesting that more than one factor was responsible for the growth stimulation.

The fatty acids myristic, arachidic and hexadecenoic were absent in some of the oils tested which elicited a dry-weight increase similar to those oils containing these fatty acids, thus these were not considered responsible for the increased growth.

Weinhold (1963), Weinhold and Garraway (1966) have demonstrated the importance of ethanol and related low molecular weight alcohols in stimulating growth and rhizomorph formation in Armillaria (Armillariella) mellea on a synthetic glucose-asparagine medium.

Ethanol, 1 - propanol and 1-butanol stimulated utilisation and rhizomorph development on a range of simple sugars and amino-acids, which otherwise produced little or no mycelial development. Alcohol also served as good sole carbon source giving growth equal to glucose plus ethanol supplement. Acetate supported some mycelial growth but no rhizomorphs.

In a study with ethanol-C^{14} Garraway and Weinhold (1968) showed that more of the radioactive carbon appeared in the lipid fraction than elswhere.

Moody and Weinhold (1972) demonstrated that a range of monocarboxylic acids, free fatty acids and plant lipids stimulated rhizomorph formation of A.mellea. The order of stimulation shown by the vegetable oils suggests that the unsaturated fatty acid fraction contributed to their activity. The effectiveness of the monocarboxylic acids and unsaturated fatty acids was shown to be proportional to chain length, toxicity of the latter increased in direct relation to the number of double bonds.

Where neutral lipids or fatty acids were sole sources of carbon, no rhizomorph development took place, indicating that they served as growth promoting substances and not sources of carbon. The organic acids and fatty acids were active at much lower concentrations than the plant lipids or triglycerides tested.

Sortkjaer and Allermann (1972) established that both

ethanol and acetate were effective in inducing rhizomorph development in A.mellea. They established a continuous requirement for ethanol. The use of disulfiraminhibited the action of ethanol but not that of acetate, suggesting that A.mellea possesses alcohol dehydrogenase activity. It was concluded/ that the pathway ethanol→acetaldehyde→acetate→fatty acids is in operation (the/ alcohols being converted to their respective acids) and which exerts a regulating effect on the metabolism of glucose.

Allermann and Sortkjaer (1973) reported vigorous rhizomorph formation and strongest growth stimulation of 2 isolates of A.mellea and an isolate of the closely related fungus Clitocybe geotropa with ethanol, 1-propanol and 1-butanol. Secondary branched alcohols, 2-propanol and 2-butanol showed only slight stimulatory capacity, primary branched alcohols, iso-butylalcohol and iso-amylalcohol exert various degrees of stimulation, tert-butyl alcohol and glycerol induced vigorous rhizomorph formation in all three isolates.

Fries (1961) showed that nonanal (pelargonaldehyde) an oxidation product of oleic acid, stimulated the growth of a number of fungi in particular wood decomposers. The closely related homologues to nonanal, the aliphatic aldehydes with 6 to 8 carbon atoms, as well as their corresponding alcohols were also active but to a lesser degree than nonanal.

Yusef (1953) reported that the growth of Polyporus schweinitzii was improved by the addition of oleic acid.

9:0 EFFECT OF OXYGEN AND CARBON DIOXIDE TENSION ON GROWTH.

Carbon dioxide markedly affects the mycelial growth of Agaricus bisporus. Data obtained on the influence of a range of carbon dioxide concentrations (Tschierpe, 1960 & 1972, Nair 1972) shows that mycelial growth is strongly inhibited by concentrations above 15% and prevented at 32%. Maintaining nitrogen constant at 79%, Nair (1972) showed that growth inhibition became significant (1% level) at 6.6% carbon dioxide. Data was obtained using a diffusion column.

San Antonio and Thomas (1972) have shown a growth optimum for hyphal fragments of A.bisporus on a synthetic agar medium at concentrations of carbon dioxide in the order 0.3-0.6%.

Le Roux (1962), Rast and Bachofen (1967) and Bachofen and Rast (1968) utilising radioactive carbon dioxide have demonstrated metabolic pathways whereby A.bisporus is capable of carbon dioxide fixation and utilisation. Le Roux (1972) has shown A.bisporus strands are better able to utilise carbon dioxide than dispersed hyphae.

Griffin and Nair (1968) using the diffusion column technique (Griffin, Nair, Baxter and Smiles, 1967) have clearly shown the effect of carbon dioxide and oxygen tension over the range of 0-15% for growth and sclerotium formation of Sclerotium rolfsii. Maximal growth was attained until oxygen concentrations lower than 4% were reached. Altering the levels of carbon dioxide alone produced almost identical results to those given by an inverse gradient of carbon dioxide and oxygen tension (nitrogen level maintained oonstant), showing that growth was roughly inhibited in direct proportion to the level of carbon dioxide in the atmosphere of the culture tubes.

Louvet and Bulit (1964) record a halved growth-rate for Sclerotinia minor at 4% carbon dioxide.

Burges and Fenton (1953) measured the growth of three soil fungi over a range of carbon dioxide concentrations from 0-20% and were able to relate the vertical distribution of the fungi by the level of growth inhibition caused by carbon dioxide. The Penicillium sp., from the upper 5 cm of soil was strongly inhibited, the Zygorrhynchus sp., abundant at 10 cm showed good growth at 20% carbon dioxide, a Gliomastix sp., present through out the soil profile was tolerant of low carbon dioxide tensions.

In general fungi colonising the aerial parts of plants or light soils are less tolerant of high carbon dioxide levels than those growing at depth of soils (Durbin 1955, 1959, Papavizas and Davey 1962).

As pointed out by Griffin (1972), however, the scope of such studies should be limited to the range 0-5% carbon dioxide, because of the unlikely occurrence of levels of this gas above this figure in the normal surface soil atmosphere, although the carbon dioxide levels in water films particularly those adjacent to respiring organs and in water saturated soils may be considerally higher (Griffin 1963). In a consideration of the role of carbon dioxide as a regulator of fungal growth in natural soils, its relative abundance in proportion to carbonate and bicarbonate levels (as determined by the dissociation constant in soils of different hydrogen ion concentration) and the affects of these ions requires to be taken into account.

Tabak and Bridge-Cooke (1968) in a thorough review on the effects of gaseous environments on fungal growth, record fungi as generally capable of vegetative growth at low oxygen tensions.

Wood-decomposing fungi including species of Schizophyllum (Schwalb and Miles 1967), Fomes (Gunderson 1961) and Pleurotus (Zadrazil 1974) may show an adaptive ability to grow at very low oxygen tensions or high levels of carbon dioxide.

10 : 0 ECOLOGICAL ASPECTS OF GROWTH AND FRUITBODY DEVELOPMENT.

Wilkins and Patrick (1940) record the fruitbody numbers of T.gambosum as the total count for six stations on clay and seven stations on chalk. Fruitbodies were collected over the period 27th April to 22nd June. Highest numbers were recorded in late May on clay sites, smaller numbers were given for the chalk sites with an earlier peak in late April and early May. Earlier fruitbody development on the chalk was correlated with a higher water content. Soil temperatures were not considered to differ significantly throughout the year between both stations.

Sporophore numbers of T.personatum are given as a total for the six stations on clay, fruiting was recorded from 22nd October to 2nd January. Wilkins and Patrick list those requirements necessary for

fruitbody production of grassland fungi as;(a), a minimum temperature above 5°C; (b), a maximum temperature below 30°C, and (c), a soil water content above 20% W/W; these requirements are only met at the times of the Spring and Autumn seasonal peaks in fruitbody production.

Grainger. J. (1946) considers soil moisture levels require to lie between 30 and 50% W/W for fruitbody development to take place, increased levels of nitrate which show maxima for April to June and September to November is also considered to promote fruitbody development during these periods. Grainger. M. (1942) considers that T. gambosum "appears with the spring maximum of nitrogen when the lower temperature appears to be sufficient for this species".

Wilkins and Harris (1946) in a consideration of the fungus floras of a Beechwood and a Pinewood report a minimum requirement of (a), a surface litter moisture content above 50% and (b), an arithmetic mean of minimum temperatures above 4°C, as the basic requirements for fruitbody formation which are only met during the respective seasonal peaks in sporophore development. Fluctuation in hydrogen ion concentration of the soils at these sites throughout the year was not determined to be sufficient to influence fungal activity, though individually selective for the fungal floras of each site.

Warcup (1951) reports isolating T. nudum mycelium in alkaline grassland at a depth of 4-5 inches, in an acid grassland the mycelium was isolated at 0-2 inches depth. Mycelium of T. nudum was also found in the litter layer of a pine forest assessed for its basidiomycete flora.

An interesting observation was that the mycelium of Tricholoma melaleucum, Clitocybe rivulosa, Crinipellis stipitarius, Stropharia coronilla and Clavaria argillacea were found associated with the roots and rhizomes of Carex glauca, Carex spp., Festuca ovina, Festuca sp., and Calluna vulgaris respectively. This suggests that grassland fungi may form a beneficial association with the roots of the associated grass species.

L.nuda is recorded as isolated from coniferous forests (Norkrans 1950; Warcup 1951; Rawald 1963; Lundeberg 1970; Lamb 1974) in mixed or deciduous forest locations (Constantin and Matruchot 1898; Votypka 1971); and pastureland soils (Warcup 1951). L.saeva has been isolated from pastureland (Wilkins and Patrick 1940; Norkrans 1950) and deciduous forest (Rawald 1962). C. gambosa has been obtained from meadow (Wilkins and Patrick 1940; Norkrans 1950, 1951; Rawald 1963) and in mixed coniferous and deciduous wood (Rawald 1962).

10:1 Mycorrhizal Associations

Trappe (1962) in a comprehensive review of probable ectotrophic mycorrhizal associations obtained from field observations, reports L.saeva as mycorrhizal with Picea abies, Pinus strobus and Quercus spp.

L.nuda is reported as forming associations with Pinus strobus, Corylus avellana, Populus deltoides and Populus nigra and with Populus nigra and Populus monilifera (Vozzo 1969).

C.gambosa is recorded as mycorrhizal with Prunus spinosa. Trappe makes the point that inability to synthesise mycorrhiza with the fungus and a range of tree spp., in vitro., does not exclude their possible formation in nature.

Lundeberg (1970) in synthesis experiments with T. nudum and Pinus sylvestris failed to establish the presence of any mycorrhizal associations. The presence of T.nudum had a clearly deleterious effect on the seedlings growth, however, the fungus was observed to cause net mineralisation of bound humus nitrogen. Inoculated together with either Boletus subtomentosus or B.variegatus one or the other fungus was dominant in the capsules, the presence B.subtomentosus was considered to reduce the extent of the inhibitory affect of T.nudum on the pine seedlings.

Norkrans (1949) was unable to synthesise mycorrhiza between

seedlings of birch or pine and T.gambosum, although growth of the fungus was reported as good.

McArdle (1932) reports mycorrhiza forming between T.personatum and the conifers Pinus strobus and Picea abies under aseptic conditions, however, Modess (1941) cast aspersions on the validity of this result and considers, together with Melin (1936) and Norkrans (1949), that T.nudum and T.personatum belong to the non-symbiotic purely saprophytic group.

Data from physiological studies on the level of cellulase and laccase activity (Norkrans 1950, Lindeberg 1946, Lundeberg 1970) is considered together with discrepances in protease and pectinase levels with a mycorrhizal fungus for T.nudum (Lundeberg 1970) to place these species firmly in the litter decomposing, saprophytic category.

Isolation of only a restricted range of higher fungi from mycorrhizal roots of a number of pine species performed by Zak and Bryan (1963), Zak and Marx (1964) and Lamb and Richards (1970) indicate that L.nuda, together with a number of other Basidiomycetes forming conspicious fruiting bodies in pine stands, may be obligate rhizoplane inhabitants or are associated with other plants in the forest community. The inability of Lamb and Richards (1970) to synthesise mycorrhizas with this fungus and pine seedlings, indicates that it is at best only a possible minor mycorrhiza former within the forest communities studied.

11:0 CULTIVATION OF FRUITBODIES.

Constantin and Matruchot (1898, 1901), Matruchot (1914), and Constantin (1942) record a number of indoor and outdoor trials concerned with fruiting T. nudum.

Spores germinated and grew readily in tubes on various leaf substrates and also in particular spent tan bark (bark of the Chestnut oak Quercus prinus). This substrate in tubes supported the development of fruitbody primordia.

Mycelium grown in flower-pots under cloches in a warm conservatory on the leaf and bark substrates, produced a "flaky, dense and crusty" growth at the surface. Fruiting took place following two months, primordia which developed were very numerous, exceeding in some cases a 100 per pot, the majority of which, however, atrophied. The largest size attained was approximately stipe height 3 cm, cap diameter 1.5 cm, in a number of cases these fruitbodies are reported to have formed gills and basidia. Fruitbody production on piles of beech leaves within the conservatory was reported to be complete and normal.

Ridge beds were constructed on the floor of a cave from beech, oak, poplar and maple leaves and also spent tan bark and a commercial mushroom growers compost. Initial inoculation was achieved by inserting clumps of leaves permeated with mycelium, obtained from that growing naturally, this was later carried out by transfering mycelium from freshly inoculated beds. Best growth was on beech leaves and spent tan bark. Fruiting took place 7 to 14 months later, generally between 10 and 12 months following inoculation. Stranding was apparent in the region where fruitbodies formed. Fruiting occurred within the cave throughout the year.

Trials outside established the perennial nature of the mycelium and fruiting capacity of the species.

Over the period 1900 to 1914 approximately eleven serial transfers from old to young beds were performed. Following this time Matruchot (1914) records abnormalities in the fruitbodies including loss of colour, a more clitocyboid appearance with a smaller more infundibiform cap bearing decurrent lamellae. A morphological abnormality which occurred throughout the trials was 'gigantism' with occasional individual fruitbodies attaining a verylarge size.

Passecker (1969) obtained fruiting of L.nuda on a partly rotted substrate, consisting of 7 parts scattered fir needles, 1 part alder leaves and 2 parts of horse manure. Inoculum was prepared by subculturing mycelium on malt agar onto a sterile horse manure filled into large containers.

Fruitbodies were obtained two months following inoculation at a room temperature of approximately 12°C and under conditions of low lighting. The fruitbodies produced were of somewhat abnormal appearance, the stipes were abnormally long and somewhat swollen in the manner of blisters, the cap was smaller, paler and infundibiform with decurrent lamellae.

Votýpka (1971) records obtaining primordia at 15°C on sterile Roe deer and horse manure substrates – which had undergone some prior fermentation – at 26 to 29 days after inoculation. The primordia achieved a maximum size of 0.5–2mm at this time, they did not develop further.

In an outside trial Votýpka inoculated a small pile of beech leaves in a sheltered position with mycelium pregrown on Roe deer manure, and obtained single fruitbodies at the end of the first and second months plus a number of smaller fruitbodies and primordia.

Duggar (1905) achieved good growth of T. personatum on leaves, soil, manure and beans by culture in test tubes. Duggar (1929) records obtaining fruitbodies of T. personatum on sterilized leaves also on fermented leaves of maple, beech and birch and on completely fermented stable manure, in the latter case obtaining well formed mushrooms two months following inoculation. Some confusion exists, however, taxonomically, from the former use of the interchangeable names T. nudum and T. personatum, from a description of fruitbody form and to references in the literature, therefore, it is possible that the former species was in fact cultivated.

Zadražil, Schneidereit, Pump and Kusters (1973) report T. nudum mycelium grew well and was resistant to infection. Details of cultivation were not given, but they report obtaining fruitbodies at a temperature of 10°C, 270 days following inoculation.

Voltz (1966) attempted mushroom house cultivation of L. nuda using a commercial compost as substrate. He reports dense, purple hyphal growth produced over the compost surface and also a mycelial mat over the surface of the casing soil, however, no fruitbody initials developed.

With the exception of Duggar's account no description of fruiting in culture of either Lepista saeva or Calocybe gambosa are available.

12:0 SUMMARY.

Optimum growth is indicated for the three species Lepista nuda, Lepista saeva and Calocybe gambosa at 25°C and 20°C for the latter species. Hydrogen ion concentrations at pH 5.0 to 6.0 are recorded as optimal.

Isolates of Lepista nuda appear to show considerable diversity in their ability to grow on a wide range of carbon and nitrogen sources. Poor growth on certain of the carbon sources may be caused by a long lag period resulting from insufficient energy to promote inducible enzyme systems required for the utilisation of the substrate. This can be circumvented by addition of 'start' glucose or may not arise where the fungus can make sufficient initial growth on other substances in the media (Lamb 1974). This may equally be the case for utilisation of nitrogen sources.

There is evidence that all three species can breakdown cellulose and lignin, although the extent of these individual enzymic capacities has not been clearly defined.

L.nuda and C.gambosa have been demonstrated to show a requirement for thiamine, this has been determined to be due to an inability to synthesise the pyrimidine molecule (Norkrans 1950). The results of Rawalds (1963) investigations, however, leave the overall vitamin requirements for the three species in an uncertain position.

Asparagine, glutamine and their corresponding keto-acids and half amides appear to stimulate growth of L.nuda and C.gambosa. There is evidence that C.gambosa may have certain deficiencies in peptide synthesis (Norkrans 1953).

All mycorrhizal synthesis experiments with isolates of L.nuda,

L. saeva and C. gambosa which proved negative were undertaken with pine seedling species, except for one instance with birch, however, these fungi are also associated with deciduous woods (possibly to a greater extent in the case of the two latter species) therefore synthesis experiments with a range of hardwood tree species requires to be undertaken before the ability to form a mycorrhizal association can be dismissed. In the absence of any mycorrhizal formation, an intimate association with plant roots in the rhizosphere of soft and hardwood tree communities and possibly grassland communities is probably important in a consideration of the ecology of these species.

Isolates of L. nuda used in the physiological studies were all obtained from coniferous locations where mycorrhizal association has been generally suspected; this fungus occupies a wide range of habitats including deciduous leaf litter and rich soils, where the fungus would be better able to maintain an independant existance. Therefore, to some extent previous investigators may have presented a one-sided representation of its physiology.

Little information on factors inducing fruit-body formation within these species is available, the fruiting of these species in spring and winter months distinct from the main autumn peak in sporophore numbers indicates individual requirements for conditions conducive to sporophore formation. The presence of Calocybe gambosa in the spring may be due to slow mycelial growth by this species, particularly at the low temperatures prevailing during the winter months, the high surface soil temperatures of spring being required to produce strong growth. It may also be that fruit-body production by this species occurs in response to a rapid rise in surface soil temperature during spring. Although such temperatures are unlikely to be directly inhibitory to growth, the fruiting response may be due to drying of the surface soil or the development of a high level of saprophytic competition in the surface soils or leaf-litter.

M A T E R I A L S

A N D

M E T H O D S

MATERIALS AND METHODS

1:0 Fruitbody Characteristics.

Fruitbodies of L.nuda and L.saeva show considerable variation in type of habitat, proportions of cap and stipe and degree of coloration, with colour fading in both in older specimens, some collections of L.nuda turning distinct russet-brown when allowed to dry out. These variations in fruitbody form and location have led to the provision by a number of taxonomists of a variety of subspecies within each group (Ramsbottom. 1951; Lange J. 1933); descriptions are more consistant for C.gambosa. The taxonomic critera of these three species has been thoroughly investigated by Singer (1962). A more concise approach to nomenclature has been recently adapted and taxonomic confines of each species more clearly defined. (Dennis, Orton and Hora, 1960).

In general the isolates collected agreed well with the descriptions of Ramsbottom (1951), Wakefield and Dennis (1950), and Watling (1973).

Variation was particularly evident in collections of L.nuda (approx.14 collections, 7 not recorded, made by author) which is considered to be a highly adaptable fungus occupying a wide range of habitats including positions in composts, leaf litters, rich soils and more exceptionally open permanent pasture land. Variation in height (3 cm to approx. 20 cm), girth and strength of purple coloration was considerable; certain isolates of L.saeva were particularly large heavy specimens. Fruitbody descriptions of Lange and Hora (1965;colour plates from J.Lange,Flora Agaricina Danica,1864-1941) for L.nuda and L.saeva were generally confusing, particularly L.nuda where only one isolate possessed a distinct red-brown cap cuticle, L.saeva possessing only blue veins from the base of the stipe fading towards the stipe apex which together with the cap lacks any lilaceous coloration. Specimens of L.nuda possess a distinct aromatic smell absent in L.saeva.

Generally descriptions of C.gambosa were similar and agreed well with specimens collected. This genus contains several tropical species (Singer,1962; Purkayastha and Chandra,1974). Its members typically possess carminophilic granules in the basidia.

TABLE: 1:0 ORIGIN OF ISOLATES

ISOLATES	DATE OF ISOLATION	LOCALITY	HABITAT	SOURCE
Lepista nuda				
1	2/72	Reading, Oxfordshire		Dr. Worgan
247.69	30/9/68	St.Hubert. S. Netherlands		C.B.S
300.58	1958	Weimar, Germany.		C.B.S
493.48	1948	Sacávem, Portugal.		C.B.S
5	10/73	Stourbridge, Warwickshire.	Grass Cuttings	
6	10/73	Stagborough Hill, Worcestershire	Permanent Pasture	
7	10/73	Pipers Hill Warwickshire	Mixed Wood	
8	10/73	Earlswood Lakes, Warwickshire.	Mixed Wood	
9	11/73	Sheffield, Yorks.	Old Flower Bed	
10	11/73	Orchard Brae, Scotland.	Under Hawthorne	Dr. Watling 1
11	1/72	Royal Botanic Gdns., Scotland.	Amongst Cinders by Gate	Dr. Watling 2
12	11/74	University Campus Birmingham.	Recently Turfed Lawn	
13	10/74	Chancet Wood, Yorks.	Mixed Wood Under Privet	
Lepista saeva				
1	1/73	Warwick, Warwickshire.	Pasture	
291.61	10/9/60	Bohem, Germany.		C.B.S
248.69	27/11/67	Longhill, Leicester.		C.B.S
355.33	1933	Germany.		C.B.S
5	10/73	Stourbridge, Warwicks.	Grass Lawn	
6A	10/73	Stagborough Hill, Worcestershire.	Mixed Wood	
6B	10/73	Stagborough Hill.	Pasture	
7	11/74	Burton-on-Trent.	Pasture	
8	10/74	Mays Wood, Warwicks.	Pasture.	

TABLE: 1:0 (cont.,)

ISOLATES	DATE OF ISOLATION	LOCALITY	HABITAT	SOURCE
Calocybe gambosa				
1	8/5/73	Great Alne, Warwickshire.		
2	5/73	Stoke Prior, Warwickshire.	Bordering Mixed Wood	
3	5/74	Moreton Bagot, Warwickshire.	Mixed Wood & Roadside Verge bordering mixed Wood.	
4	5/74	Droitw ich, Worcestershire	Roadside verge, bordering mixed Wood.	
5	5/74	Thornhill Wood, Little Alne, Warwickshire.	Grass bank bordering mixed Wood.	

1. Watling Herbarium No. 10123. C.B.S.-Central bureau voor
2. Watling Herbarium No. 8896 Schimmel Cultures, Baarn, Netherlands.

P L A T E 1.

Sporophores of Lepista nuda obtained growing wild.

The older flanking specimens from the same collection have turned reddish-brown (russet) upon drying (Isolate No.7).

The central specimen indicates the original light lilaceous colouration (a pinkish overtone has been introduced in reproduction of the photograph). (Isolate No.8.).

SCALE 2 cm.

P L A T E 2.

Sporophore of Lepista nuda of stout proportions with small deep purple cap and thick lighter coloured stipe. Growing within a deep pile of beech leaves.

SCALE 1 cm.

P L A T E 3.

Very robust sporophores of Lepista nuda with the typical 'tricholomatoid' inverted cap. Virtual absence of colouration in the gills and speckled lilaceous stipe Growing amongst mixed deciduous leaves at the bottom of a garden.

SCALE 1 cm.

P L A T E 4.

Lepista nuda sporophores of relatively delicate proportions from compost pile. Purple colour more strongly developed in the gills, light colour on the stipe.

SCALE 1 cm.

PLATE 5.

Robust sporophore of Lepista saeva. Purple colour developed in veins at the base of the stipe fades rapidly after collection.
(Isolate 5.)

SCALE 1 cm.

P L A T E 6.

Sporophores of Calocybe gambosa. Obtained from well-rotted leaf humus in roadside verge.
(Isolate No.4).
Cap surface with reddish tints.

P L A T E 7.

Sporophores of Calocybe gambosa. Obtained from grassy bank. Distortion of cap by grass roots during emergence producing typical 'gamboid' (hoofed) appearance.
(Isolate No.5).

SCALE WIDTH OF COIN 2.8 cm.

The cleft appearance of the fruitbody is a distinct feature of this species.

Species in the genus Lepista typically possess a sordid to cream pink spore print, the spores have a thin, hyaline finely roughened spore wall, characters which have formed the basis for its delimitation from the closely allied genera Tricholoma and Clitocybe.

C.gambosa is a vernal species, L.nuda and L.saeva fruiting in late autumn and winter, the former (in Scotland) fruiting in quantity after the first frosts (Watling, personal communication). The time and duration of fruiting is highly dependant on the temperature and rainfall of the individual season, generally C.gambosa was collected in late May and did not appear as early as 23rd. April (St. Georges Day), fruitbodies of L.nuda and L. saeva were collected as late as February in the mild winters of 1974 and 1975.

1:1 Taxonomy.

Classification according to Dennis, Orton and Hora (1960) 'The New Check List'. With the exception that Calocybe gambosa is used in preference to Tricholoma gambosum.

Lepista nuda (Bull.ex Fr.) Cooke		(the 'wood blewit')
Syn:	Tricholoma nudum	Bull.ex Fr.
	Rhodopaxillus nudus	(Fr.ex Bull.) Maire
Lepista saeva (Fr.)	Orton	(the 'pasture blewit')
Syn:	Tricholoma personatum.	Fr.
Agaricus saevus Fr.	Lepista personatum.	(Fr.ex Fr.) Cooke
	Rhodopaxillus saevus	(Gill.) Maire
Calocybe gambosa (Fr.) Sing.		(the 'St Georges Mushroom').
Syn:	Tricholoma gambosum	(Fr.) Kummer.
	Lyophyllum gambosum	(Fr.) Sing.
	Tricholma graveolens	(Pers ex Fr.) Kummer
	Tricholoma albellum	(DC.ex Merat) Kummer
	Tricholoma georgii	(L. ex Fr.) Quél.
	Calocybe georgii	(L. ex Fr.) Kühner
	Lyophyllum georgii	(L. ex Fr.) Kühner & Romagn.
	Agaricus prunulus.	Vitt. non Scupoli Fr.

2:0 CULTURE OF ISOLATES.

Tissue blocks were taken from pileus tissue at the general apex of the stipe following the aseptic procedures outlined by Watling (1971) and half submerged in fresh 2% malt extract agar (Pantidou 1961). Hyphae generally grew well from the aerial portion of the tissue block. Where the material was not fresh (as in the case of L. saeva which tended to take-up water in the field) generally aureomycin 2-6 mg/L (or occasionally streptomycin or penicillin) was incorporated into the agar. With C. gambosa which was initially slow growing, benomyl (Taylor 1971) was added at 5mg/L to suppress possible phyco-and ascomycete contaminants.

Cultures were maintained on 2% malt agar slopes in 20 ml capacity McCartney bottles at 4°C.

3:0 STUDIES IN PETRI-PLATES.

9cm sterile disposable polystyrene Petri-plates were employed. Approximately 20 ml 2% Boots malt extract agar was used for routine culture work. Exact 20 ml quantities of media were introduced for the experimental studies.

Inoculation, at the centre of each plate was carried out with a No. 3 corkborer (6mm dia.,) mycelium agar plugs were taken from 1 cm within the periphery of 3-4 week old colonies. Excess staled agar was cut from beneath the mycelial disc to enable rapid growth down on to the new medium and minimise carry-over of staling products.

Temperature studies were conducted as an assessment of the rate of mycelial extension for 4, 3 and 1 isolate of L. nuda, L. saeva and C. gambosa respectively, maintained in incubators or fridges at the stated temperature. Colony diameters at right angles were measured and averaged for two replicates at each temperature.

Assessment of p-diphenol oxidase activity on gallic and tannic acid media was made for a range of L. nuda(13), L. saeva(7) and

and C.gambosa (2 isolates) following the procedures outlined by Davidson, Campbell and Blandell (1938) and Lindeberg (1946). Gallic and tannic acids were millipore filtered (0.44/mm membrane pore dia.,) and added at concs., of 850 mg/L and 500 mg/L to glucose-asparagine (to provide a clear medium for faint reactions) and 2% malt extract agar media respectively. 6 mm and 18 mm mycelium agar inocula were employed, one and two inocula per plate for gallic and tannic acid media respectively, plates in replicates of three.

Growth and gross colony morphology for one isolate each of L.nuda and L.saeva and C.gambosa was assessed on glucose-asparagine basal agar medium with the inclusion of a range of lower aliphatic acids and fatty acids for duplicate plates at concentrations of 0.5, 1 and 5 g/L. Methods of sterilisation and addition followed those for the procedures given in a later section on stationary culture in flasks, the organic acids and lipids being added and dispersed in the cooled mediumjust prior to pouring.

Growth and clearing of 11 isolates of L.nuda, 6 isolates of L.saeva and 2 isolates of C.gambosa was determined on a modified Norkrans Cellulose medium (appendix) which contained 1% 72 hr ball-milled cellulose finely dispersed to give a white opaque medium (Eggins and Pugh, 1962). Growth and cellulolytic ability was measured as mycelial extension and development of clearing along 2 diameters at right angles for duplicate plates.

All isolates were assessed for growth on malt agar following isolation, leading to selection of those strains giving best growth for more extended studies.

Three isolates of L.nuda, 2 of L.saeva and 2 of C.gambosa were further grown in triplicate on potato dextrose, chlamydospore and Eggins and Pugh Cellulose media,to check for stranding and rhizomorph formation as reported by Voltz (1966) for his strain of L.nuda on chlamydospore agar, and observed to be formed by certain isolates during the course of studies on cellulose media.

3:1 Studies with divided Petri-plates.

Sterile disposable polystyrene Petri-plates with a single vertical partition extending to approximately two thirds the height of the Petri-plate base were employed.

The principle consisted of establishing a vegetative and reproductive substrate in the two halves of the plate (Hume and Hayes 1972). Agars were added in volumes of 20 ml per side, the former was always 2% Boots malt agar solidified with 1.5% Oxoid agar No. 3. The vegetative medium was the experimental substrate, this consisted of either (a) 1.5% water-agar, (b) Skermans carbon free medium agar (Skerman 1959), (c) Soil extract agar, (d) a sterile peat/chalk mix (approx., proportions chalk/peat/water 1:2.3:5 $^{w}/_{w}$) containing 25% $^{v}/_{v}$ granulated activated charcoal (Long 1970), (e) a sterile water agar/granulated actived charcoal mix (in proportions, 3:1 $^{v}/_{v}$, total volume 20 ml). These media were employed for three isolates of each of the species L.nuda (7, 8 & 13) L. saeva (248.69, 5 &6B) and C.gambosa (1, 3 & 5) in duplicate plates.

Inoculation was achieved by two equally spaced 6 mm agar inocula on the malt agar medium. Incubation at 25°C continued until the fungus had overgrown approx., one third of the experimental substrate (3-4 weeks), at this stage the plates were transfered to cellar (16°C) or room (20°C) temperature. Where mixed bacterial 6 mm water agar plugs were employed these were placed in pairs on the water agar (medium (a)), equally spaced, opposing the mycelial inoculation plugs.

For the incubation at the lower temperature a dry enviroment was sought to reduce the relative humidity and condensation of water vapour within the Petri-plate. This phase of incubation was extended upto two months duration.

4:0 ISOLATION OF BACTERIA AND MIXED INOCULATION PROCEDURES.

Ninety-nine bacteria isolates were recovered from dilution plates made on nutrient and soil extract agars, of five soils obtained from

Coleshill, Birmingham (permanent pasture supporting a fairy-ring of C.gambosa) Moreton Bagot, Stagborough Hill, Droitwich and Evington Meadows. The isolates were characterised by morphology though not identified to the generic level (with the exception of 8 fluorescent pseudomonad sp., determined on King's Medium B). Three strains of L.nuda (5, 7 & 8), L.saeva (248.69, 5 & 6B) and C.gambosa (1, 3 & 5) were assessed for growth inhibition caused by these isolates, by radially streaking the isolates out from a central c. 3 cm fungal colony on malt extract agar.

The effects of cell-free filtrates of mixed bacterial flora from the five individual soils plus a sixth 'casing-soil' sample from a currently cropping mushroom bed of Agaricus bisporus was assessed by millipore-filtration of the combined bacterial isolates from each soil, grown-up in 100 ml quantities of soil extract medium for 3 days as a shake flask culture, as 2, 4 & 6 ml additions of the filtrate to 20 ml of soil extract agar. Growth was assessed by single isolates of L.nuda (247.69), L.saeva (248.69) and C.gambosa (1) from 1.8 cm mycelial malt agar inocula placed centrally in the Petri-plate. The plates were in triplicate with soil extract medium as control.

4:1 Preparation of Soil Extract Agar.

The soil extract medium consisted of: Soil extract stock soln., 100 ml, KH_2PO_4 0.5g., Glucose 1.0g Agar 15g., Tap water 900 ml.

The stock soln., of soil extract was prepared by steaming 1kg of soil from Evington Meadows, Leicestershire, (Clay loam) with 1 litre of tap-water in an autoclave for 30 mins., This was mixed and dispersed to two 1 litre measuring cylinders and 2.5g calcium carbonate added to each and allowed to stand for one hour. The supernatant liquid was poured-off and was repeatly filtered through a double layer of Whatman No. 1. filter paper until clear. This was then autoclaved at 15 p.s.i. for 20 minutes and maintained sterile by aseptic addition to the agar medium. The stock soln., was held in the cold store at $4^{\circ}C$ for further use.

5:0 GROWTH IN FLASKS.

6 mm inocula obtained and treated as given in the previous section were placed on fresh 2% malt agar plates and incubated at 25°C for 24-48 hrs. to enable the formation of aerial hyphae, which greatly facilitated floating the inocula on the liquid medium (Norkrans 1950). Selection of only those inocula with abundant aerial hyphae also ensured uniformity of inocula throughout the experiment and reduced the initial lag period of growth.

100 ml wide-mouth Pyrex Erlenmeyer flasks were used throughout, stoppered with non-absorbent bleached cotton-wool and containing 25ml of the synthetic liquid medium. Certain of the experiments were incubated in a standard gravity convection incubator at 24°C ± 1°C, the larger experimental systems were incubated at room temperature (22°C±2°C). Generally flasks were incubated for 28 days occasionally shorter or longer, as indicated, and once dry-weights were recorded at 7 day intervals. With the exception of C/N ratio, pH and leaf extract studies (duplicate flasks), all experiments were conducted with replicates of 3 flasks per treatment. Flasks and associated glass-ware were cleaned in Pyroneg (Diversey Products) -a phosphate based detergent - and rinsed finally with several changes of deionised water.

For assessment of growth, mycelial mats were drained on a fine wire/ mesh supported over a Buchner funnel and flask maintained under suction. This enabled the combined culture fluids to be collected and pooled, the mesh was then transferred over a sink and the mats washed with deionized water contained in a wash bottle. The drained mycelial mats were then transferred to previously dried and tared 7 cm. dia., filter-paper discs or in later experiments tared squares of aluminium foil. The mats were oven dried at 65°C to constant weight and weighed to the nearest tenth of a milligram on a Mettler balance. The use of aluminium foil enabled the dried mycelium to be readily removed for analysis and also further minimised errors in the final weighing by eliminating uptake of water which is otherwise absorbed by the dried filter-paper disc.

Growth was assessed where ball-milled cellulose and calcium oxalate were used as carbon sources, by measurement of total Kjeldahl N (Jacobs 1965), and conversion to dry-weight by comparison with dry-weight and nitrogen values of mycelial mats formed by growth with glucose as carbon source.

The basal medium used for stationary culture in flasks was a modified Norkrans glucose-asparagine medium adjusted to pH 6.0 with appropriate quantities of m/15 pot. dihydrogen orthophosphate and disodium hydrogen orthophosphate as indicated in a later section dealing with pH. This resulted in a phosphate concentration at this pH of 0.00614 Molar. Initial experiments as indicated contained only 1g asparagine/L. The adaption of ferric citrate as iron source instead of ferric chloride was found to considerably improve growth in initial trial experiments. The level of thiamine was increased from 50 to 200μg to allow sufficient quantity for the greatly increased yields obtained in these experiments to those of Norkrans (1950,'53), and a biotin addition was made as certain strains indicated at least a partial requirement for this substance from the vitamin studies.

The basal medium was composed of: Glucose 20g., Asparagine 2g., M/15 kH_2PO_4 131.5 ml and M/15 Na_2HPO_4 18.5 ml to give pH 6.0., $MgSO_4.7H_2O$ 0.5g., NaCl, 0.1g., Ferric citrate 5mg (Iron stock soln. contains 1g Ferric citrate ($\equiv$ 0.17gFe) and 0.64g Citric acid per 100 ml) Zn $SO4.7H_2O$ 4.4mg., $CaCl_2.2H_2O$ 0.1g., Thiamine 200μg Biotin 5μg Distilled water to 1 litre. pH 6.0 best approximated to the optimum pH for strains of L.nuda, L.saeva and C.gambosa from pH studies.

Sterilisation of carbon and nitrogen sources wherever possible was carried out by millipore filtration using a cellulose acetate filter of 0.44μm pore size. Where media required to be autoclaved this was achieved at 15 p.s.i. for 5 minutes to keep time in the autoclave to a minimum. Generally individual media components were sterilised in 200 ml medical flats in a small stainless steel autoclave with a rapid cycle time. Sterilised materials were pipetted into flasks, tubes and bottles using

standard aseptic procedures. Millipore sterilisation and individual autoclaving procedures were essential to prevent decomposition of medium components, the production of inhibitory substances in the media (Cochrane 1958), and the generally concurrent changes in pH and exhaustion of buffer capacity.

All chemicals employed in these studies were of 'Analar' or 'puriss' grade.

6:0 GROWTH STUDIES. pH ADJUSTMENT AND STERILISATION.

6:1 Determination of Optimum pH.

Growth over the pH range 2 to 8 was assessed for 3 strains each of L.nuda and L.saeva plus a single strain of C.gambosa utilising the glucose-asparagine synthetic medium containing 1g asparagine/L. Incubation was at 25°C for 21 days. Individual quantities of media were made-up and adjusted to unit values within the stated pH range by combination of the Sorensen phosphate buffers m/15 KH_2PO_4 + N/10 HCI, M/15 kH_2PO_4 +M/15 Na_2HPO_4 according to the table of Lindeberg (1939). Actual pH values for each quantity were determined by a combined glass pH electrode and meter, these were then sterilised by millpore filtration and 25 ml quantities aseptically pipetted into oven sterilised Erlenmeyer flasks.

Hydrogen ion concentration of the pooled culture solutions for the 3 replicates was taken following growth of the isolates for 3 weeks to determine pH shift. This method was found to be the only solution to circumvent the considerable alteration to the media or components of it which occur with autoclaving, and the concurrent shift in pH which is particularly marked in the alkaline range.

6:2 Carbon: Nitrogen ratio

Assessment of the optimum C/N ratio was conducted for a single strain of L.nuda and L.saeva. Concentrations of asparagine were chosen at 0.1 (0.1864 gN/L) and 0.3 per cent (0.5592 gN/L) at carbon:

nitrogen ratios for spacings of 3 units over the range 9 to 28:1, by altering the level of glucose addition in each series. Duplicate flasks for each strain at the individual ratios were harvested every 7 days over a 28 day period and the dry-weights and pH of the pooled culture fluids recorded.

Values for mycelial dry-weights and pH of the pooled culture fluid following 28 days incubation were obtained for single isolates of L.nuda, L.saeva and C.gambosa cultured in four series of C:N ratios at individual nitrogen concentrations of 0.00932, 0.01864, 0.03728 and 0.05592 per cent. C:N ratios within each series were achieved by alteration of the carbon level. Carbon and nitrogen concentrations were obtained by manipulation of glucose and asparagine concentrations within the standard basal liquid medium. Values are for duplicate flasks.

6:3 Vitamin requirement.

The vitamin requirement was determined for 2 strains each of L.nuda, L.saeva and C. gambosa utilising the basal medium plus vitamin addition. Asparagine added at 1g/L was purified by treatment with finely divided Norit activated charcoal according to Yusef(1953) in an attempt to remove vitamin sources.

The vitamin sources and yeast extract were sterilised by millipore filtration. The growth requirement was assessed by removal of individual vitamin sources from the complete vitamin mix which contained inositol, choline chloride and lipoic acid as additional substances in an attempt/ to simulate the composition of yeast extract.

All vitamin sources were added at a concentration of 200μg/l with the exception of biotin (5μg), choline chloride (200mg) and lipoic acid (20 mg). P-aminobenzoic acid is part of the folic acid moity. Yeast extract was added at 0.4 g/L. The glucose and asparagine were millipore sterilised and added in appropriate concentration to the prior autoclaved basal salt soln., made-up to the requisite concentration.

6:4 Utilisation of Carbon Sources.

Single strains of L. nuda, L. saeva and C. gambosa were cultured on 32 single carbon sources and 11 combinations of carbon sources at a concentration of 8g C/L: raffinose, starch, inulin and dextrin additions were corrected for loss on drying. Asparagine was added as nitrogen source at 2g/L. The nitrogen source for C. gambosa consisted of 19 amino-acids added in equal quantities of nitrogen as given for the amino -acid mix in the following section. This was added to and autoclaved with the basal salt soln., plus vitamins at 15 p.s.i. for 5 minutes.

All soluble carbon sources were pH adjusted to 6.0, millipore sterilised and added to the prior autoclaved basal salt solution. Insoluble carbon sources were autoclaved with the basal salt soln., for 5 minutes at 15 p.s.i. Asparagine was filter sterilised and added separately. Autoclaving resulted in some hydrolysis of the inulin to fructose and possible partial hydrolysis of starch and pectin to their constituent sugars. Flasks containing insoluble carbon sources required pH adjustment to 6.0 following autoclaving. Flasks were incubated at room temperature ($22^{\circ} \pm 2^{\circ}C$) for 28 days (L. nuda and L. saeva) and 32 days (C. gambosa).

6:5 Utilisation of Nitrogen Sources.

Growth was determined for two isolates each of L. nuda, L. saeva and C. gambosa on a range of complex organic nitrogen sources, ammonium, nitrate and nitrite nitrogen. Nitrogen souces were added at a concentration of 0.3728 gN/L equivalent to 2g asparagine/L. All nitrogen sources were pH adjusted to 6.0 then individually filter sterilised and added separately together with millipore filtered glucose to the prior autoclaved basal salt and vitamin solution.

Growth of single strains of L. nuda, L. saeva and C. gambosa was assessed for 19 amino-acids as sole nitrogen sources plus the amides asparagine and glutamine. Growth in a synthetic amino-acid mix which contained the 19 amino-acids in equal proportions of nitrogen and on casein hydrolysate was also determined. All nitrogen sources individual and combined were added at 0.3728g N/L except as indicated, and pH adjusted

to 6.0 prior to sterilisation. Sterilisation, due to limited quantities available of many of the amino-acids, was achieved by individual autoclaving at 15 p.s.i. for 5 minutes.

Amino-acids were generally added as the L-isomeric form, racemic forms of alanine, methionine, valine and phenylalanine were added at double the concentration of nitrogen on the assumption that only the L-form is utilised (Reusser, Spencer and Sallans, 1958 b). L-cystine and L-tyrosine were largely insoluble and DL-β-phenylalanine partly soluble at pH 6.0.

6:6 Effect of Fatty and Organic acids on growth.

The effect of a range of saturated and unsaturated fatty acids and organic acids on growth of single strains of L.nuda, L.saeva and C.gambosa was determined in the synthetic glucose-asparagine medium containing 1g asparagine per litre. Linoleic, oleic, palmitic and stearic acids (100 and 500 mg/L) were diluted indiethyl ether and added to the glucose-salts soln., in flasks, prior to autoclaving. Acetaldehyde and acetic acid (50 and 100 mg/L) which effect the cellulose acetate membrane filter if filter sterilised were also added directly to the flasks prior to autoclaving.

Ethanol (100 and 500 mg/L) propionic, iso-valeric and buteric acids (50 and 100 mg/L) were diluted, millipore filtered and pipetted into the autoclaved, glucose-salts medium. Asparagine was added separately following filter sterilisation. All treatments were in replicates of three. The flasks were incubated at 24°C for 28 days. Following autoclaving (during which the diethyl ether was dispelled) the fatty acids tended to form droplets on the surface of the medium.

6:7 Effect of Ash and Metal ions on growth.

The importance of various elements in the basal salt soln., was approximately assessed for two strains of L.nuda and L.saeva.

Additions were made to a basal soln., which contained: KH_2PO_4 and $Na_2H\ PO_4$, KCL , $MgSO_4 \cdot 7H_2O$ and ferric citrate plus thiamine and biotin vitamin additions at concentrations similar to those for the synthetic glucose-asparagine medium, potassium chloride replacing sodium chloride. Ash additions were made at 0.4 g/L. The synthetic mineral soln., was formulated as a synthetic yeast extract ash and contained per litre: $CaCl_2 \cdot 2H_2O$ 0.5 mg , $(NH_4)_6\ MO_7\ O_{24}$ 0.23 mg , $CuSO_4 \cdot 7H_2O$ 0.5 mg , $MnSO4 \cdot 7H_2O$ 0.49mg , $CuSO_4 \cdot 7H_2O$ 0.59 mg , the amounts and composition being similar to that given by Coscarelli and Pramer (1962) with alterations to allow for a smaller quantity of nutrient medium per flask and the use of sulphates not oxides of manganese and zinc.

The leaf and manure preparations were obtained as given in a later section. Malt extract and yeast extract werepowdered commercial preparations. The ash was obtained by ignition at 450°C for 18 hours in a muffle furnace. The lower temperature prevents the destruction of potassium. Ash preparations were solubilised in small quantities of conc.HCL (Coscarelli and Pramer, 1962). Zinc oxide and zinc oxide plus calcium chloride additions to the basal salt soln., were made at concentrations given for the synthetic mineral soln.. This treatment was also extended to two strains of C.gambosa where the nitrogen source consisted of DL-valine, DL-methionine, L-glutamic, L-aspartic acid, L-β-phenylalanine,

L-serine, L-tryptophan and L-cystine at equal quantities of nitrogen totalling 0.3728 g N/L. This combination was formulated as a substitute for the amino-acid mix used in nitrogen source studies. However, very little growth took place and the results are not included, it is concluded that this combination of amino-acids is very inhibitory to growth of C.gambosa (this was also the case for certain amino-acid formulations assessed by Norkrans, 1950).

6:8 Growth responce to leaf and manure extracts.

The growth responses of single isolates of L.nuda, L.saeva and C.gambosa were determined for the addition of a range of 3 animal manures and leaf extracts from 5 deciduous and 4 coniferous trees to the basal glucose-asparagine medium containing 1g asparagine per litre. The leaf and manure extracts were added at concentrations of 0.5, 1 and 3 mg dry matter per ml according to Melin (1946). Figures were obtained for the mean of 2 replicates at each concentration, flasks incubated at 24°C and harvested following 21 days of growth.

Freshly fallen leaves were collected in autumn, pure leaf collections being made for each tree species. The leaves were rinsed and blended to fragment them to small size. The leaf macerates together with the animal manures were 72 hr ball-milled in a 4% soln.,. Dry-weights were taken for 10 ml samples following ball-milling and the extracts finally sterilised by autoclaving at 15 p.s.i. for 1 hour. This treatment enabled the extracts to be readily pipetted into flasks and the finely divided form provided availability of any growth stimulatory substances that existed in the solid and liquid components.

7:0 CLEARING IN CELLULOSE COLUMNS.

The celluloytic ability of 9 isolates of L.nuda, 7 isolates of L.saeva and 2 isolates of C.gambosa was assessed using the cellulose column technique of Rautela and Cowling (1966).

This was basically modified by using the 72 hr. ball-milled

cellulose medium of Eggins and Pugh (1962). The phosphate buffer combination at pH 6.0, as used in the basal medium, replaced the potassium dihydrogen orthophosphate of the cellulose medium. The technique involves measuring the rate of clearing down a cellulose column beneath the fungus mat. The clearing is clearly visible against the milk-white opaque agar.

Cellulose concentrations of 0.25 and 1% were used, 15 ml of medium dispensed into 20 ml capacity screw-cap McCartney bottles. It was necessary to agitate the medium (cooled to 45°C) thoroughly, then plunge the tubes into ice, to ensure the particles of cellulose were uniformly distributed throughout the final cellulose agar column. The use of screw-caps, partly loosened, permitted the tubes to be incubated at room temperature over two months without drying and shrinkage of the agar; this is an inherent problem where the standard boiling-tubes of Rautela and Cowling plugged with cotton-wool are used. The tubes for each strain were in replicates of 5.

8:0 GROWTH IN TUBES.

Growth rates on solid substrates were assessed using glass tubes of 10" length and $1\frac{1}{4}$" width, plugged at both ends with bleached non-absorbent cotton-wool. This arrangement enabled the tubes to be more evenly packed throughout, than would be the case with test-tubes and furthermore allowed for free gaseous exchange along the length of the column.

Growth on a commercial 'short-straw' mushroom compost (approx. 2.3g% N and 36% dry-matter) was recorded with 130g compost packed as evenly as possible into the tubes, for 9 isolates of L.nuda, 5 isolates of L.saeva and 2 isolates of C.gambosa.

The tubes were autoclaved for 1 hour at 10 p.s.i. on 3 separate days, inoculated with a 1.8 cm agar-mycelium disc, and capped with approx., 1" of a sterile thoroughly moistened peat-chalk mix to prevent drying-out of the agar - this in effect acted as a standard casing layer in which development of strands and possible initials was most obvious.

Overall growth-rate was determined in mm/day, together with an empirical assessment of mycelium density and development of strands for 3 replicates of each strain.

8:1 Growth in flasks.

Growth in 250 ml wide-mouth Erlenmeyer flasks containing 60g autoclaved compost (treated similarly to that given for tubes), plus an addition of 8g autoclaved peat-chalk casing following spawn-run, was assessed for 5 isolates each of L.nuda and L.saeva. Theflasks were inoculated with 1.8 cm mycelium-agar discs and spawn-run at 25°C for 1 month. Extent of stranding and indications of possible formation of initials in the casing were recorded as an extension of the results from growth in tubes.

9:0 FRUITBODY DEVELOPMENT ON AGAR.

Cultures of 3 isolates of L.nuda, L.saeva and C.gambosa were assessed for fruitbody formation on a natural agar medium at 12°C with a Circadian light cycle of 9 hrs light 15 hrs dark supplied by 'daylight' fluorescent lamps.

100 ml quantities of 2% malt extract agar supplemented with 1g/L yeast extract was dispensed into 250 mlwide-mouth Erlenmeyer flasks and the fungus cultures inoculated centrally with 6 mm inocula, 5 replicates per strain, and allowed to overgrow the agar surface for 3 weeks at 25°C. The appearance of stranding, possible primordia and fruitbodies was noted over a period of 5 months.

9:1 Fruitbody development on solid substrates.

Six 5 kg quantities of commercial mushroom compost were spawned at a 50:1% ratio with 3 strains of L.nuda and L.saeva and each transferred to 4 containers (dimensions: 6" square, slightly tapering to 7" depth) at 1.25 kg per container. 3 further 10 kg amounts of compost in plastic bags were spawned with the isolates of L.saeva.

A prior prepared rye grain spawn of these 6 isolates was used as inoculum. Spawn-run continued for 32 days at *c.* 25°C following which the compost was fully permeated with mycelium except in the bag containing *L. saeva* 248.69, which was discarded. The spawn-run compost in the bags was transferred to six containers, 1.67 kg amounts to each.

The containers were cased with *c.* 1½" layers over the separate halves of the spawn-run compost surface with 4 combinations: loam soil and standard peat chalk casing, pear and oak leaves, beech and birch leaves, sitka spruce and larch leaves, a treatment for each container. These materials had received a prior fumigation with methyl bromide for 3 days. The 6 additional containers for the two isolates of *L. saeva* were equally subdivided and cased entirely with commercial peat-chalk casing or loam soil, these containers did not receive any further casing treatment. Containers were initially covered with a layer of plastic, including during spawn-run, to maintain high humidity and moist conditions in the surface layers, and incubated at approximately 16°C.

Following 40 days the containers received a 2nd casing treatment; at this stage the *L. nuda* isolates had produced a very strong growth through the leaf and soil layers with formation of a dense surface lilaceous layer. Growth of *L. saeva* isolates was generally weak through the leaf layers though considerably stronger through the soil. The second casing consisted of an additional layer of clay loam and peat-chalk separately positioned on the four quarters of the mycelium surface, checkerboard fashion, laid thin centrally increasing in thickness towards the corners of the container. Incubation continued at approximately 16°C under conditions of intermittent dim fluorescent lighting for a further 60 days, with regular watering to maintain the casing material moist.

Records were made of those isolates fruiting and individual containers dissected to determine the number of fruitbodies forming and their individual stage of development within the container.

9:2 Preparation of Grain Spawns.

100g quantities of grain (cereal grains, rye, wheat, milo or millet) in 500 ml Erlenmeyer narrow-neck flasks, were initially washed in a stream of cold water to remove dust, chaff and split grains, and then drained. To the individual flasks was added 30 ml water and 1.65g $CaSO4.2H_2O$ plus 0.42g $CaCO_3$. The grains were heated to boiling on a hot-plate and allowed to stand at room temperature for 24-48 hrs., followed by autoclaving at 15 p.s.i. for 1½ hrs in order to thoroughly cook the grain.

It is necessary to frequently shake the grain during heating, before, and after cooling to thoroughly disperse the chalk and gypsum and thereby prevent sticking of the grains.

Boiling followed by incubation, prior to autoclaving, eliminates a number of contaminants with resistant spores. The flasks were plugged with non-absorbent cotton-wool and capped with aluminium foil following boiling, which prevented excessive wetting of the bungs during extended autoclaving and reduced water-loss from the grain during cooling. The volume of water added to the wet grain was calculated to give a final moisture level in the grain of about 40-50 per cent. The quantities of chalk and gypsum used give a final pH of about 6.5 (Stoller 1962).

The grain was inoculated with 5-8, 1.8 cm mycelial-agar discs from cultures grown on 2% malt agar. The flasks were further shaken about a week following inoculation to disperse newly colonised grain, and again following incubation (4-6 wks. at room temperature) in order to break-up the spawn and thereby prevent excessive intergrowth and tissueing within the grain mass.

10:0 EXPERIMENTS WITH THE GAS DIFFUSION COLUMN.

The diffusion column technique of Griffin, Nair, Baxter and Smiles (1967) was used. The column differed somewhat from the type detailed by them, was constructed in brass 72" x 5" diameter and fitted with 17 horizontal parts in duplicate along its length which allowed sampling of specific gas concentrations throughout the range employed. The individual ports were designed with two pairs of vertically opposed outlets and a single terminal horizontal outlet. In later experiments the top outlets were made serviceable by extending them with the use of thick plastic tubing and retaining the specimen tubes on two racks disposed along the length of the column. Silicone rubber 'suba-seals' were inserted into one set of the horizontal outlets to enable direct sampling from the individual ports. This arrangement allowed for a maximum of 9 culture vessels per gas concentration.

Specimen tubes (1" x 6") were used as culture vessels each containing 20 ml glucose-asparagine agar or 2% malt agar, laid flat to form a thin layer along the length of the tube. 2% agar addition was used in order to give the agar sufficient strength. The tubes were inoculated centrally with 6mm inocula and colony extension measured along the agar surface at regular intervals. Replicates were in two or three for each species at the individual gas concentrations, as indicated.

Gas was supplied from commercial cylinders of nitrogen (containing c. 0.3% oxygen), oxygen and carbon dioxide, plus the use of atmospheric air. Where carbon dioxide was required to be removed from the air, it was passed through 10% NaOH and washed through distilled water. Standard gap meters were used to regulate the volumes of gas passing to the displacement vessels and where necessary calibrated by displacement of water.

The column was allowed to stabilise between 8-12 hrs prior to use. At steady-state, the gas concentration at any position x, along the cylinder, is determined by the formula

$$Cx = Cm - \frac{x}{l} (Cm - Cn)$$

where l is the length of the cylinder and Cm and Cn are the individual gas

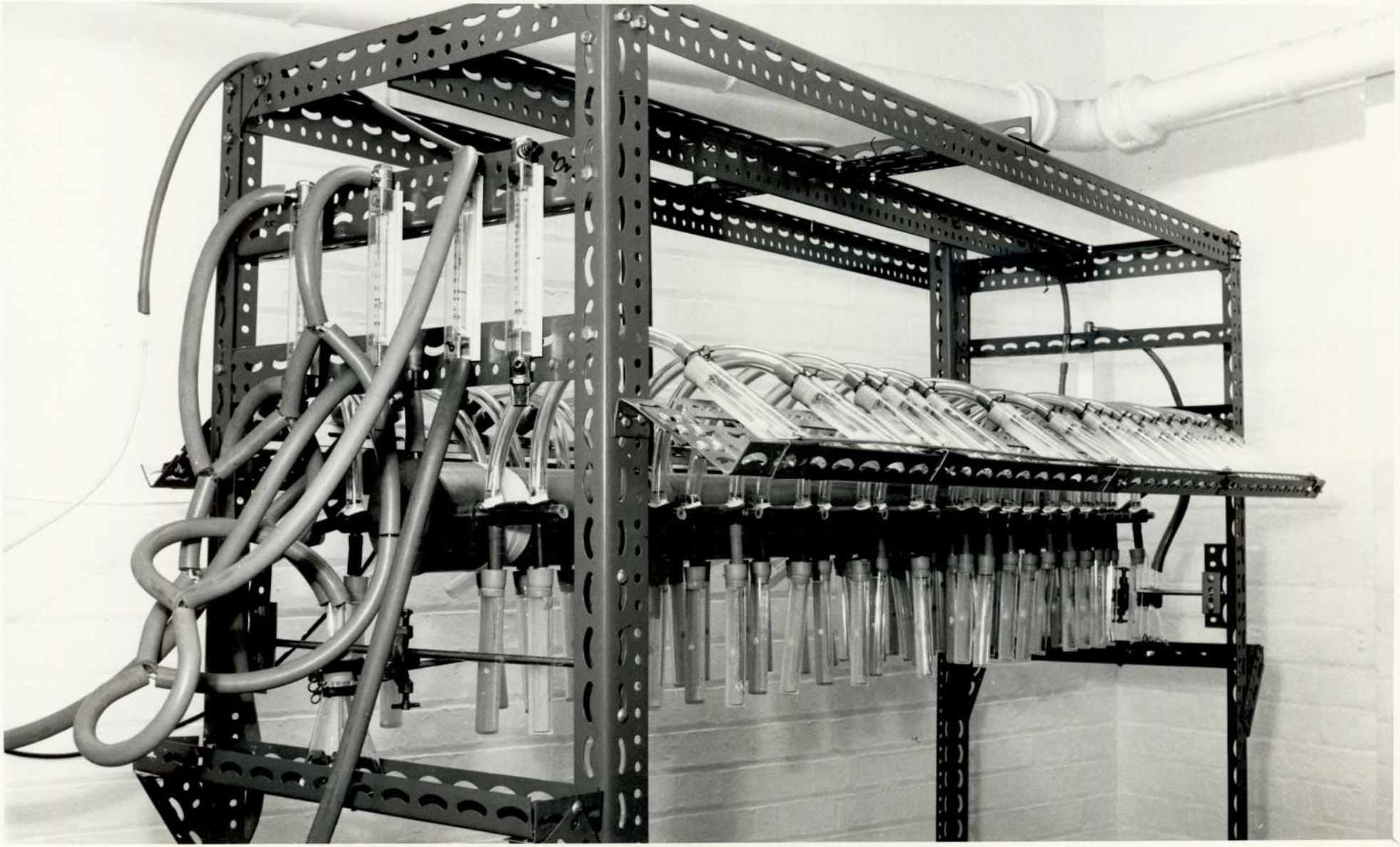
concentrations in the displacement chambers at either end of the cylinder.

A calibration curve was obtained over the range of carbon dioxide concentrations 0-1.0% by use of a Fisons Mini gas chromatograph. The values obtained agreed well with theoretically derived values (Figure 15.6).

Individual experiments were designed to measure the growth response in the oxygen range 0.3-21.0% and carbon dioxide ranges 0-5.0% and 0-12.0%

PLATE 8.

GAS - DIFFUSION COLUMN.

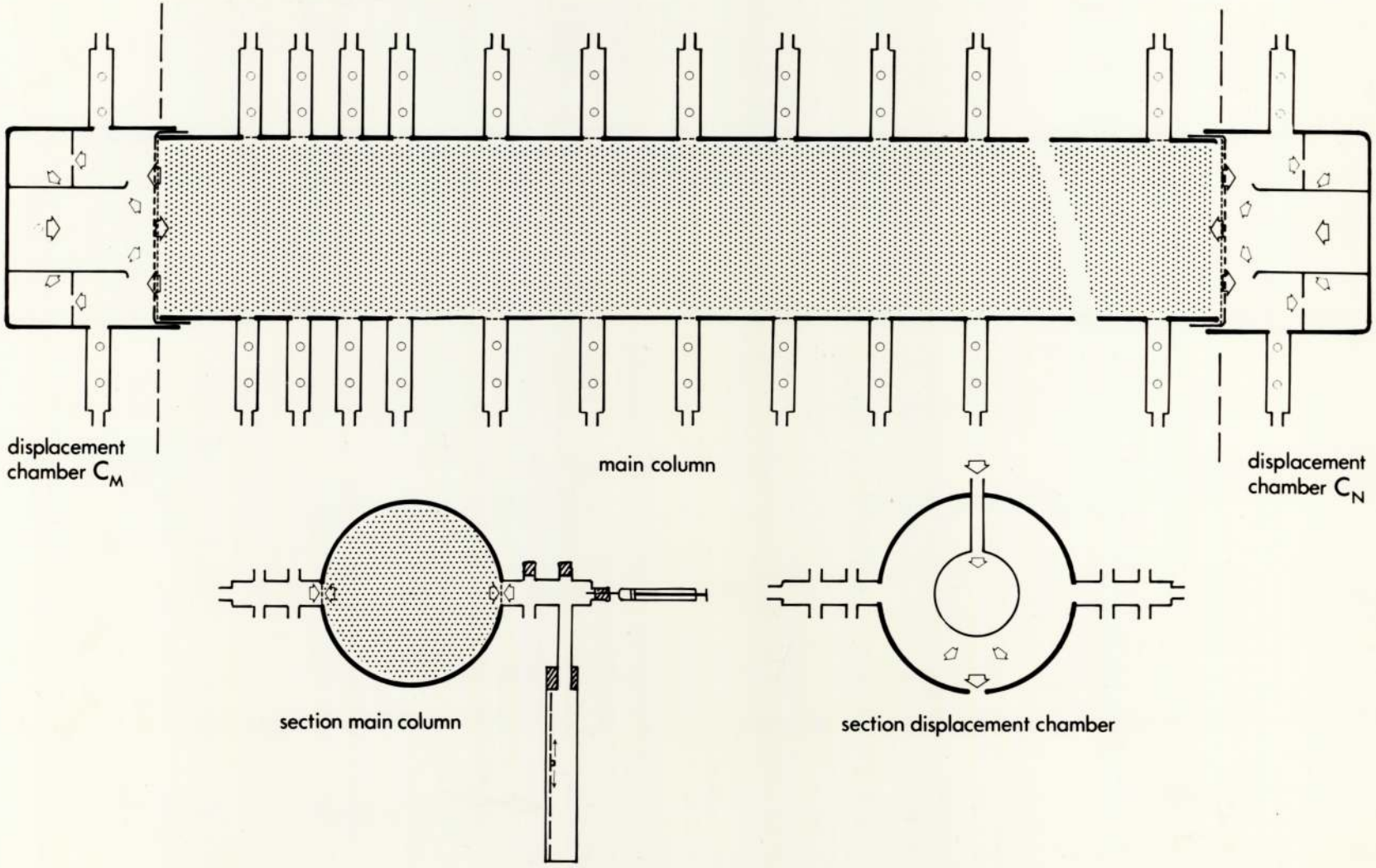


P L A T E 9.

DIAGRAMMATIC SECTION

THROUGH GAS-DIFFUSION

COLUMN.


displacement chamber C_M
main column
displacement chamber C_N
section main column
section displacement chamber

11:0 STATISTICAL ANALYSIS OF RESULTS

11:1 Derivation of Least Significant Difference from Analysis of Variance and Standard Error of the Mean.

Least Significant Difference (L.S.D.) was determined by:

L.S.D. = $\sqrt{(2)}$ x t x S.E.M. where Standard Error of the Mean.

$$(S.E.M.) = \sqrt{\frac{\text{error mean square}}{\text{number of samples for each mean.}}}$$

t = Students t at 5.0, 1.0 and 0.1 per cent. Levels of significantce with $n - 1$ Degrees of freedom (^{o}F)

11:2 Standard Error of the Mean (S.E.M.) Determinations.

Obtained from the formula:

$$S.E.M. = (\varepsilon) = \sqrt{\frac{\sum (m-M)^2}{N(N-1)}}$$

11:3 Analysis of Variance Determinations.

(Bishop 1966)

Calculations of Error Mean Square were determined from:

(1) Analysis with three or more replicated treatments.
(2) Analysis with two experimental factors and replication.

R E S U L T S

SECTION 1 RESULTS

1:0 COLONY CHARACTERISTICS

Lepista nuda

Lepista nuda possessed hyphal cells of long, thin, dimensions with a diameter of c. 3 μ (Modess 1941); cells possessed a regular distribution of clamp-connections at each septum.

Isolates of L. nuda were readily produced from pileus and stipe tissue blocks in a fresh condition. The hyphae grew readily from the upper portion of the block to give a largely aerial, loosely woven colony with a distinct violaceous tint. A strong purple colour was produced by aggregation and compression of the aerial hyphae such as occurred following manipulation with a needle or removal of a sample with a cork-borer. Following three to four mycelial transfers the colonies became considerably less aerial. Cultures obtained from the Central Bureau voor Schimmelcultures, Netherlands, isolated in 1948 and 1958 were producing adpressed and submerged colonies on agar media.

Regularly subcultured colonies of L. nuda produced stable and consistent growth, generally of a fine and dispersed nature though this character varied to some extent over the range of isolates. Sectors or secunds were not produced during normal culture conditions, although light tufts of aerial hyphae and in some instances compact areas of deeply lilaceous hyphae did arise where nutritional exhaustion or drying-down of the agar medium was taking place. In general none of the isolates readily produced strands on agar media even following extended periods of incubation. The instances where stranding did take place were; (a), following extended incubation on cellulose agar; (b), following growth in flasks on cellobiose media; (c), following growth in flasks, containers, tubes and divided Petri-plates on compost or peat-chalk media, (d), following growth from individual grains of rye grain spawn onto 2% malt agar (for a single isolate No. 8).

Voltz (1966) reported rhizomorph development by his strain of L. nuda on chlamydospore agar, however, this finding could not be repeated with 3 strains of L. nuda on this medium.

Lilaceous colour development in cultures of L. nuda was

largely in response to light although some isolates (5, 7, 8 and 10) produced stronger colouration or were prone to sporadic production of dark colouration, those cultures maintained in a dark incubator or in a coldstore at 4°C in darkness were generally white or sporadically coloured compared to the more even development of colour with colonies maintained under conditions of natural or artificial lighting. This was also the case with respect to the L. saeva isolates. Strong spawn growth of isolates in flasks and containers through the surface peat/chalk casing layer resulted in strongly coloured purple, tissued mycelium at the casing surface.

Growth of grain-spawns was generally good. Once colonised the mycelium became increasingly denser, this could lead almost to the development of tissueing, where cultures were maintained in the coldstore over long periods of time (this was particularly strong for one strain, No. 13).

Good growth was produced by all strains on 2% malt extract agar(Boots Pure Drug Co.) (MEA), more luxuriant growth resulted when this was supplemented with either casein hydrolysate (CMEA), mycological peptone (PMEA) or yeast extract (YMEA) at 1g/litre. Growth of these isolates on potato dextrose agar (Oxoid Ltd.) and chlamydospore agar (formula of Voltz 1966) was very poor, resulting in brown discolouration restricted aerial development and on chlamydospore agar submerged development (probable effects of the very low level of nitrogen in these media).

Growth of isolate 1 on Treschow Wort agar (Treschow 1944), Lutz (Singer 1962) medium and Hagem Malt agar (modified by Modess 1941) was generally inferior to malt agar. Growth of a variety of L. nuda isolates on the latter was very restricted, poor growth was probably due to unavailability or direct inhibition by the iron source.

Lepista saeva

Hyphae of L. saeva were wider and more branched than those of L. nuda, clamp connections were clearly observable on individual septa. Hyphae grew readily from tissue blocks, however, isolates of this species were considerably less aerial and more compact,

PLATE 10.

Strong strand development by Lepista saeva. Isolate 6B following growth on malt/yeast extract agar in flasks.

SCALE 1 cm.

forming denser and slower growing colonies, though of a very uniform overall appearance.

Development of purple-lilaceous colouration was particularly strong only in some colonies, in these, however, the colonies were darker, possibly due to denser development of the surface hyphae. An isolate maintained in culture for a long period (C.B.S. 355.33) developed adpressed surface growth.

Stranding generally developed on substrates similar to those described for L. nuda. L. saeva isolate 6B grown in flasks on YMEA produced very strong development of strands, this was particularly so where in several of the flasks, perpheral points of inoculation (produced accidentally) developed strongly and linked with the centrally developed colony arising from the inoculation plug.

Growth of grain spawns following colonisation became very dense, in particular where these were maintained at 4°C in the coldstore, the density of development produced purple tissued regions, tissueing took place strongly at the surface of the grain spawn. Such spawns could not be readily broken-up and removed from the flask. Colonies produced by isolates 248.69, 5 and 6B in stationary liquid culture were particularly dense.

L. saeva cultures were generally more susceptible to staling with increasing colony age and at temperatures higher than optimal.

Colonies produced by isolates 5 and 6B on potato dextrose agar were brown and restricted, chlamydospore agar produced very light surface to sub-surface development. Growth of L. saeva isolate 1 on Treschow Wort agar, Lutz and Hagem agars was generally inferior to 2 % malt agar. Malt agar supplemented with 0.1% Casein hydrolysate, peptone or yeast extract afforded heaver colony development.

Calocybe gambosa

Hyphae of C. gambosa were heterogeneous in size though wider than those of the Lepista spp.,. Hyphae under magnification

frequently appeared aggregated into strand-like formations.

On a few natural media isolates 1 and 2 particularly the former developed a 'bushy' appearance where the aerial hyphae appeared to be composed of short 'asbestos-fibre' like strands, these out-growths in particular arose from the inoculum plug where autolysis or staling of the media had taken place. Growth of C. gambosa isolate 1 as wheat-grain spawn produced these spiked 'bushy' out-growths. Newly isolated mycelia particularly strains 3, 4 and 5 produced submerged hyphal developement. Isolates 1 and 2 were routinely cultured, newly isolated hyphae grew very slowly, a large number of mycelial transfers were required to enable adaptation to the medium and ensure, uniform and repeatable colony growth. For this reason the later isolated strains 3, 4 and 5 were not used regularly. Irregular culturing resulted in long lag periods before growth was resumed.

C. gambosa was prone to the occassional formation of rapidly growing sectors. This occurred with a number of flasks receiving acetic, butyric or valeric acid supplements to the standard glucose-asparagine medium in stationary liquid culture and on occasional Petri-plates where the additions were the fatty-acids, oleic, stearic and palmitic acid.

All strains produced a yellow pigment on 2% malt agar and particularly strongly on chlamydospore agar. The culture fluid from growth on the glucose-asparagine medium was generally bright yellow-orange. The production of pigment varied with the carbon or nitrogen source used in stationary culture work. Freshly isolated strains noteably 3, 4 and 5 produced large numbers of orange stellate crystals over the surface of the agar medium particularly on rich media. Isolates 1 and 2 produced very light development on chlamydospore agar but good development on potato dextose and malt extract agars. Favourable growth with these media indicates good utilisation of starch, but also possibly that a high C/N ratio is favourable to growth. Strong development with these media may also reflect the presence of growth stimulating substances (e.g. amino-acids or peptides) within the media. Growth on unamended glucose-asparagine medium was generally poor.

TABLE 1:1 Comparison of Growth of single Isolates on a range of Solidified Media.

Natural/Semi-synthetic Media	LEPISTA NUDA 1 mm/day	LEPISTA SAEVA 1 mm/day
HAGEM MALT AGAR (Modess 1941)	0.95	1.0
TRESCHOW WORT AGAR (Treschow 1944)	0.81	2.46
LUTZ MALT AGAR (Singer 1962)	1.63	2.23
MALT EXTRACT AGAR (MEA)	2.28	3.53
MALT EXTRACT + CASEIN HYDROLYSATE AGAR (CMEA)	2.54	4.33
MALT EXTRACT + YEAST EXTRACT AGAR (YMEA)	2.3	4.21
MALT EXTRACT + PEPTONE AGAR (PMEA)	2.59	4.45
NUTRIENT AGAR (NA)	1.8	1.08

Yeast Extract, Casein Hydrolysate, Peptone supplements at 1g/L to 2% Malt extract Agar.

Figures average of 5 replicates for 2 colony diameters at right angles per colony.

1:1 Combined Studies in Divided Petri-Plates and in Mixed Cultures

Inhibition of fungal colony extension by sirgle bacterial isolates from each soil was due to a certain proportion of bacteria within the spectrum isolated. The effect was most evident with isolates from the lighter sandy soils (Coleshill, Droitwich and Stagborough Hill) particularly in C. gambosa strains than for isolates obtained from the heavy clay soils (Moreton Bagot and Evington Meadows).

Rapid, light mycelial development occurred over the soil extract medium, in general, addition of cell-free solutions of the five natural soils strongly inhibited growth off the inoculaton plug particularly the 4 and 6 ml additions. The L. saeva isolate was tolerant of the lower level of addition of the cell free solution. The solution obtained from the'casing-soil' flora was considerably less toxic in its effect, it produced no growth inhibition of the L. saeva isolate.

Treatments (a), (b), (c), (d), (e) and the use of mixed bacterial inoculation plugs, had no direct morphological effect on growth (barring some inhibition of growth in the vicinity of the bacterial inoculation plug). Colonisation was light, though somewhat stronger and more uniform with treatments (c) and (d). Only L. nuda isolate 13 strongly colonised all treatment halves. Light strand development occurred with L. nuda isolates on (b) and (d), isolate 13 formed a strand network on medium (e). L. saeva isolate 6B formed light stranding on medium (d). Development of strands generally originated and was strongest in the aerial hyphae passing over the intermediate dividing wall of the Petri-plate. Stranding was also often obvious radiating from the inoculation plug on the malt agar section of the plate.

SECTION 2

2:0 EFFECT OF HYDROGEN ION CONCENTRATION ON GROWTH

L. nuda isolates showed a pH optimum at approximately 7.0 with a shift over the 21 day incubation period to more acid conditions in

EFFECT OF HYDROGEN ION CONCENTRATION ON GROWTH

3 WEEKS INCUBATION AT 25°C

FIGURE 2.1

pre-incubation

post-incubation (pH shift)

Lepista nuda 7

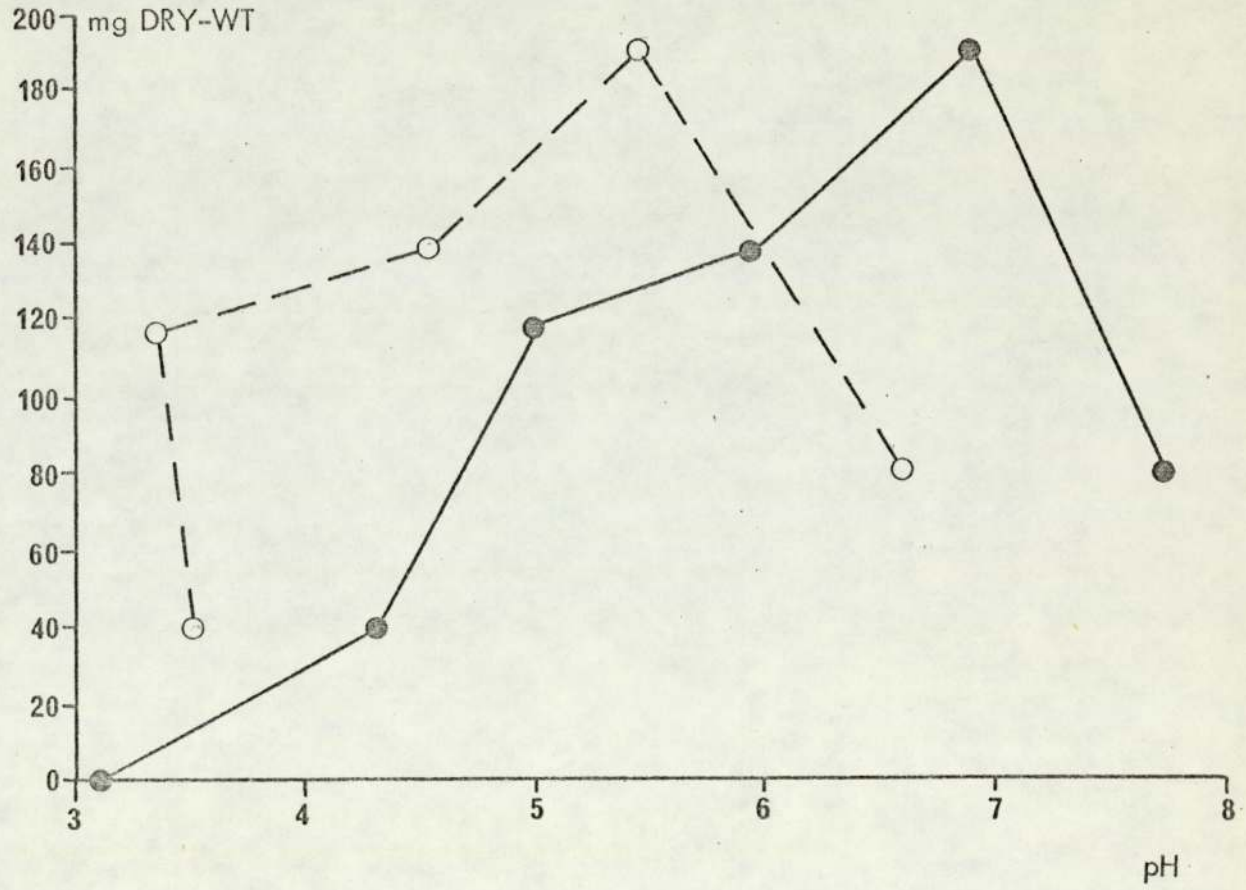


FIGURE 2.2

EFFECT OF HYDROGEN ION CONCENTRATION ON GROWTH

Lepista nuda 6

3 WEEKS INCUBATION AT 25°C

pH CULTURE FLUID
—●— pre-incubation
--○-- post-incubation
(pH shift)

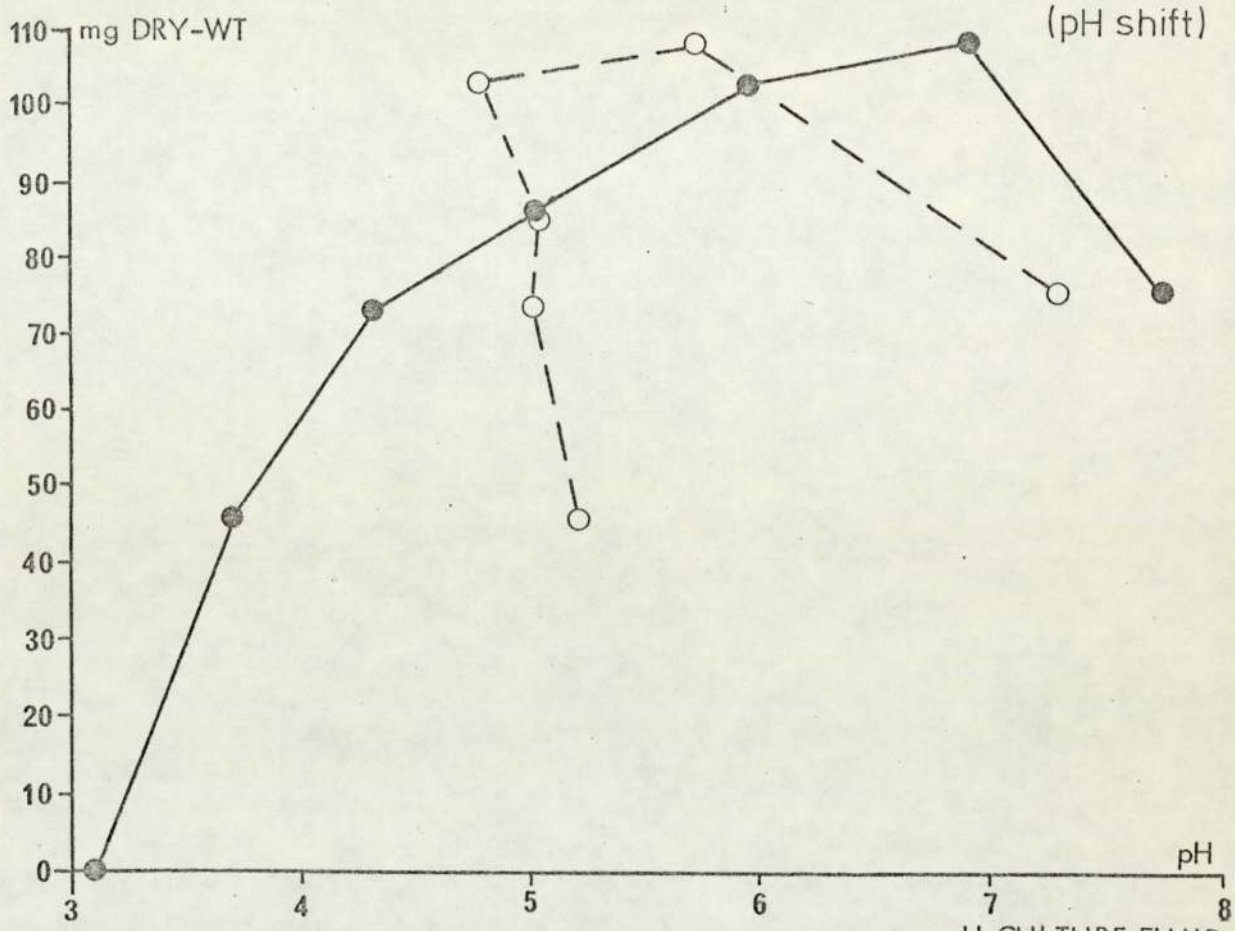
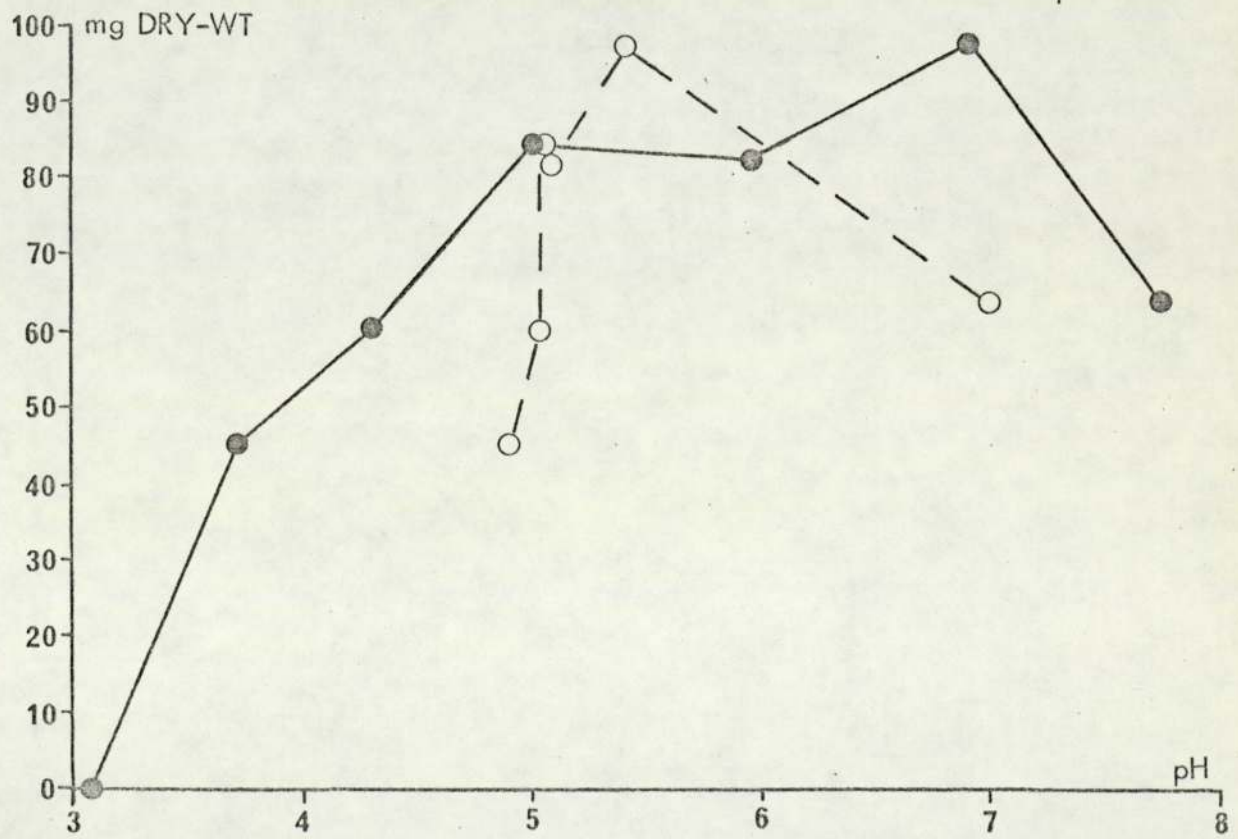


FIGURE 2.3

Lepista nuda 8

3 WEEKS INCUBATION AT 25°C

pH CULTURE FLUID
—●— pre-incubation
--○-- post-incubation
(pH shift)



EFFECT OF HYDROGEN ION CONCENTRATION ON GROWTH

3 WEEKS INCUBATION AT 25°C

FIGURE 2.4

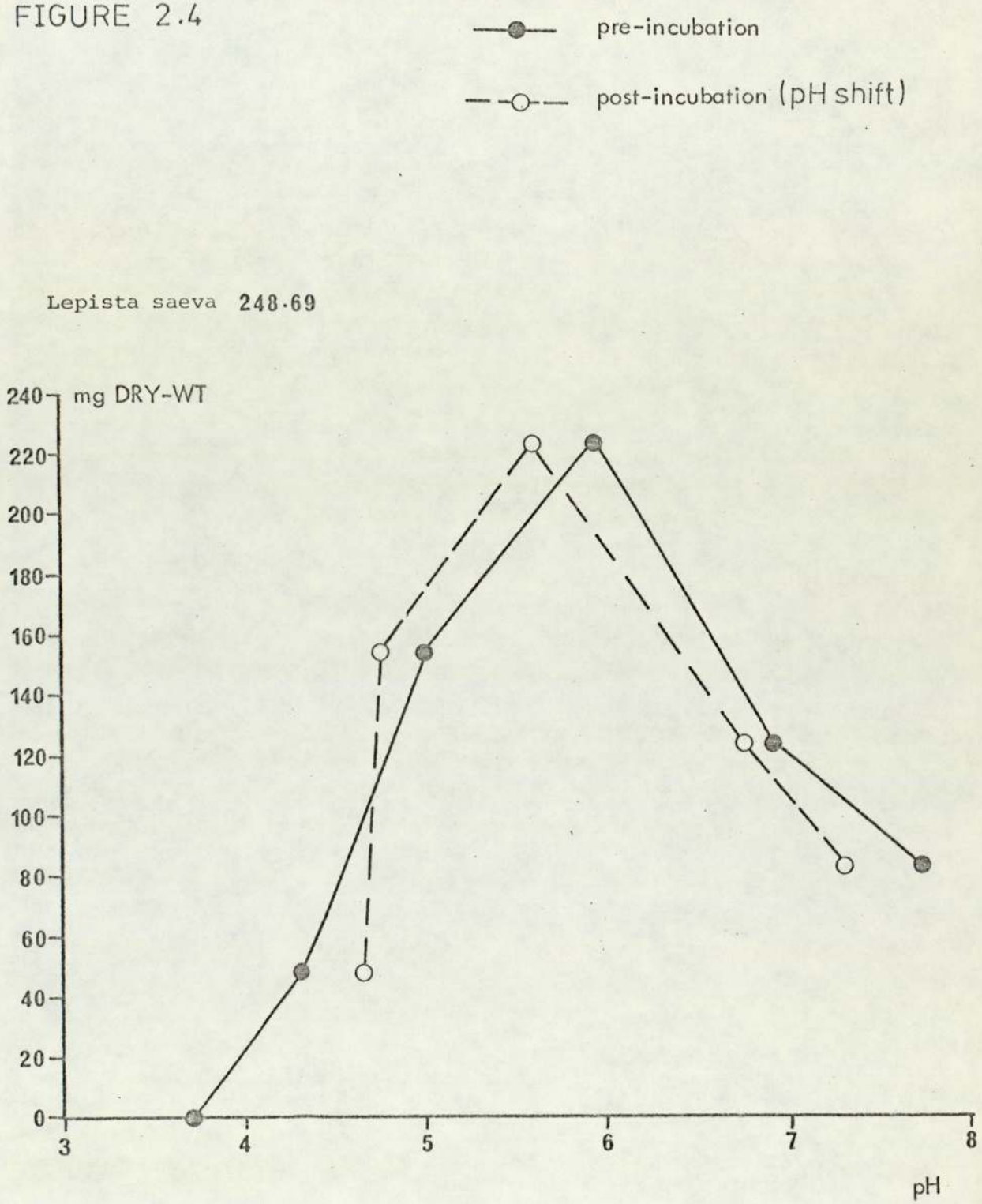


FIGURE 2.5

EFFECT OF HYDROGEN ION CONCENTRATION ON GROWTH pH CULTURE FLUID

3 WEEKS INCUBATION AT 25°C —●— pre-incubation

—○—post-incubation

(pH shift)

Lepista saeva 5

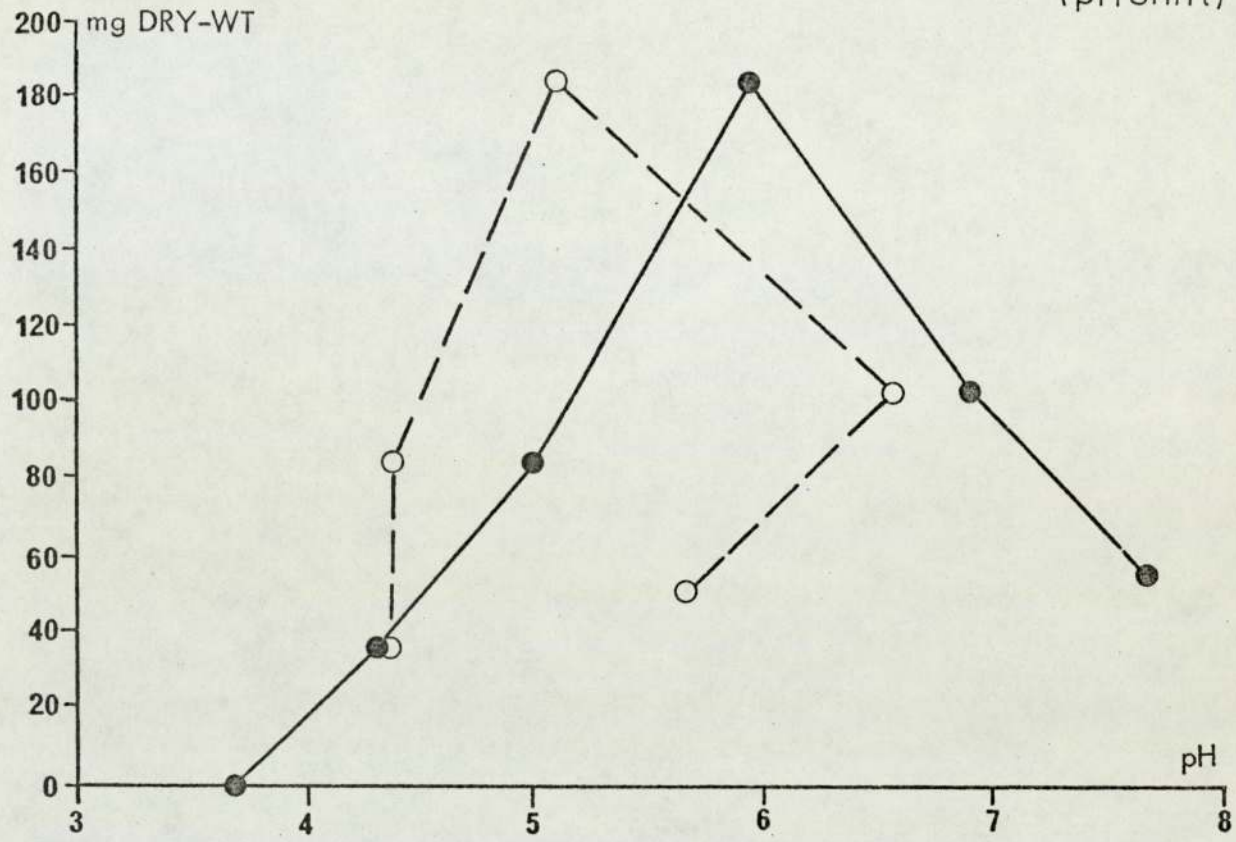


FIGURE 2.6

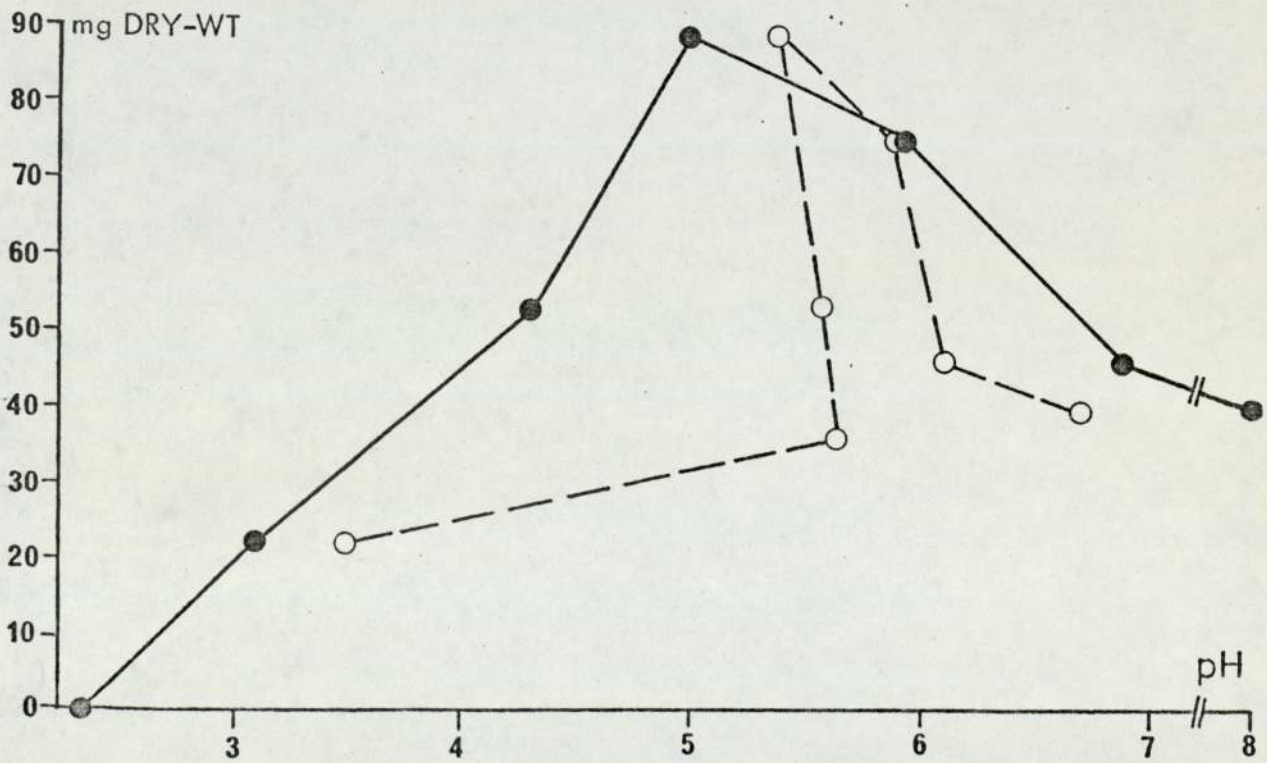
pH CULTURE FLUID

3 WEEKS INCUBATION AT 25°C —●— pre-incubation

—○—post-incubation

(pH shift)

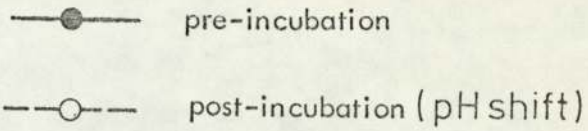
Lepista saeva 6A



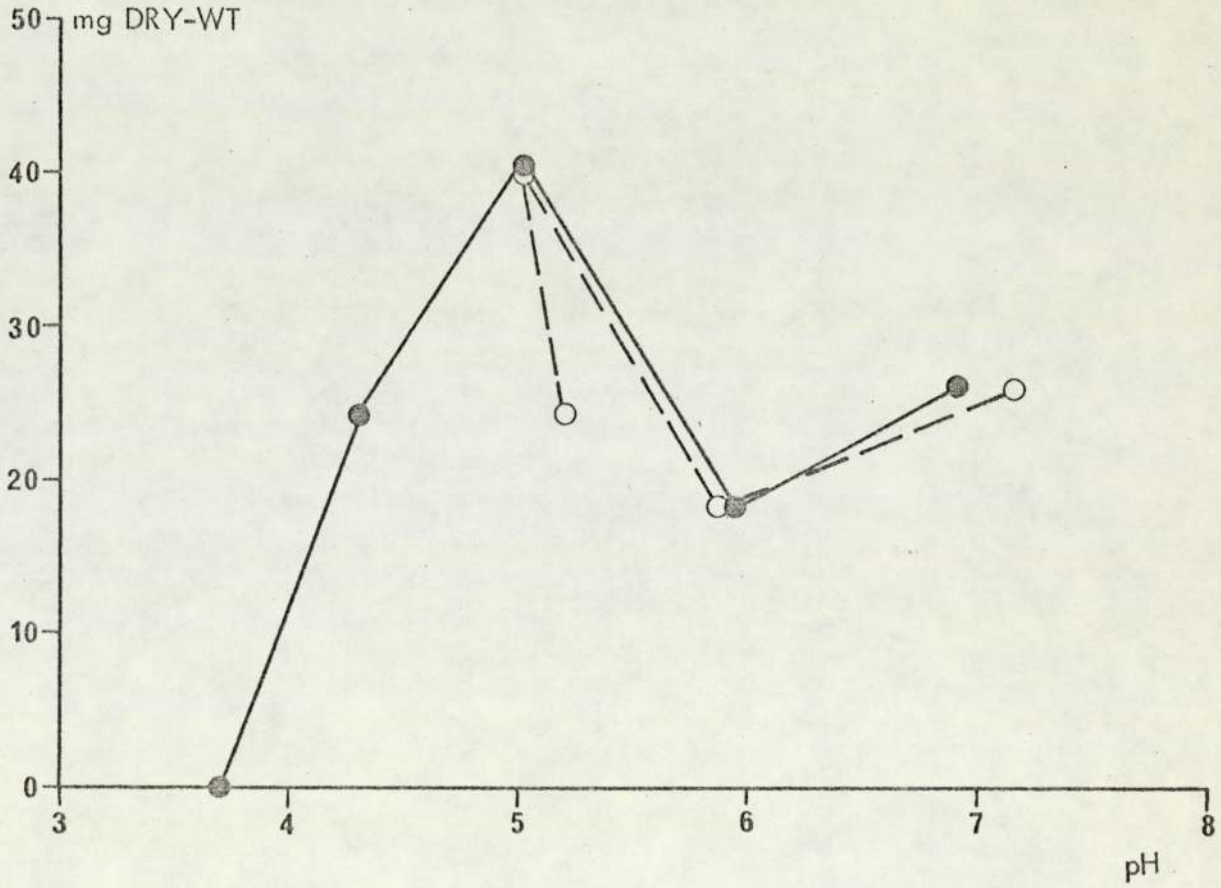
EFFECT OF HYDROGEN ION CONCENTRATION ON GROWTH

3 WEEKS INCUBATION AT 25°C

FIGURE 2.7



Calocybe gambosa 1



the region of pH values 5.0 to 6.0 (Figures 2.1, 2.2 and 2.3). Isolates 6 and 8 were more tolerant of acid conditions than isolate 7, growth, however, was only prevented for all isolates at pH 3.1. Growth was not strongly inhibited in the alkaline range due probably to a rapid shift in pH to more favourable acid conditions. In general there was a shift in pH values to terminal values in the range 5.0 to 7.0.

L. saeva isolates indicated a more distinct optimal value within a narrow zone of pH preference between 5.0 to 6.0 (Figures 2.4, 2.5 and 2.6). The alteration in pH values following incubation was not as marked as L. nuda. Isolates were less able to tolerate acid conditions, growth for 2 isolates (248.69 and 5) was inhibited at pH 3.7, however, isolate 6A was able to tolerate values down to 2.35.

Growth of a single isolate of C. gambosa (2) (Figure 2.7) was optimal at pH 5.0. Growth was inhibited at pH 3.7. The pH shift was generally to more alkaline conditions.

The growth of all three species was good in these millipore filtered solutions where the formation of inhibitory substances or breakdown products frequently a product of media autoclaving was prevented. Excellent growth of the C. gambosa was made in millipore filter sterilised solutions.

3.0 EFFECT OF TEMPERATURE ON GROWTH

Of the four isolates of L. nuda used in this study two demonstrated an optimum at 25°C, two at 20°C (Figure 3.1). Those isolates with the lower temperature optima grew better at 4, 10 and 15°C. All isolates were inhibited by a temperature of 30°C.

Growth of the three isolates of L. saeva tested was optimal at 20°C (Figure 3.2). Good growth was generally attained at temperatures of 10 and 15°C. Slight growth of all isolates occurred at 4°C. Growth of all isolates was inhibited at 30°C.

Temperatures of 27 to 28°C had a marked effect on growth

FIGURE 3.1

EFFECT OF TEMPERATURE ON GROWTH
21 DAYS INCUBATION

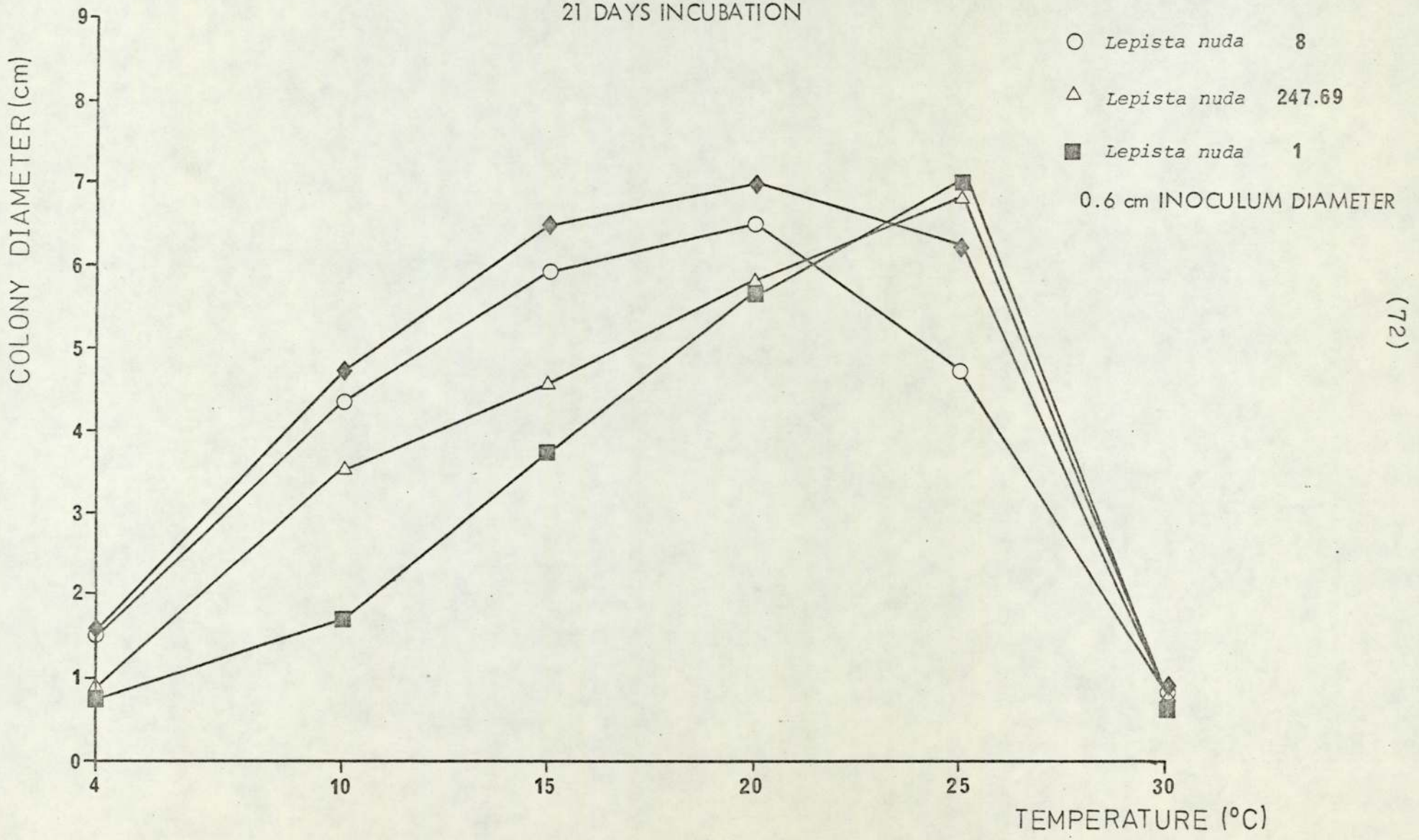


FIGURE 3.2

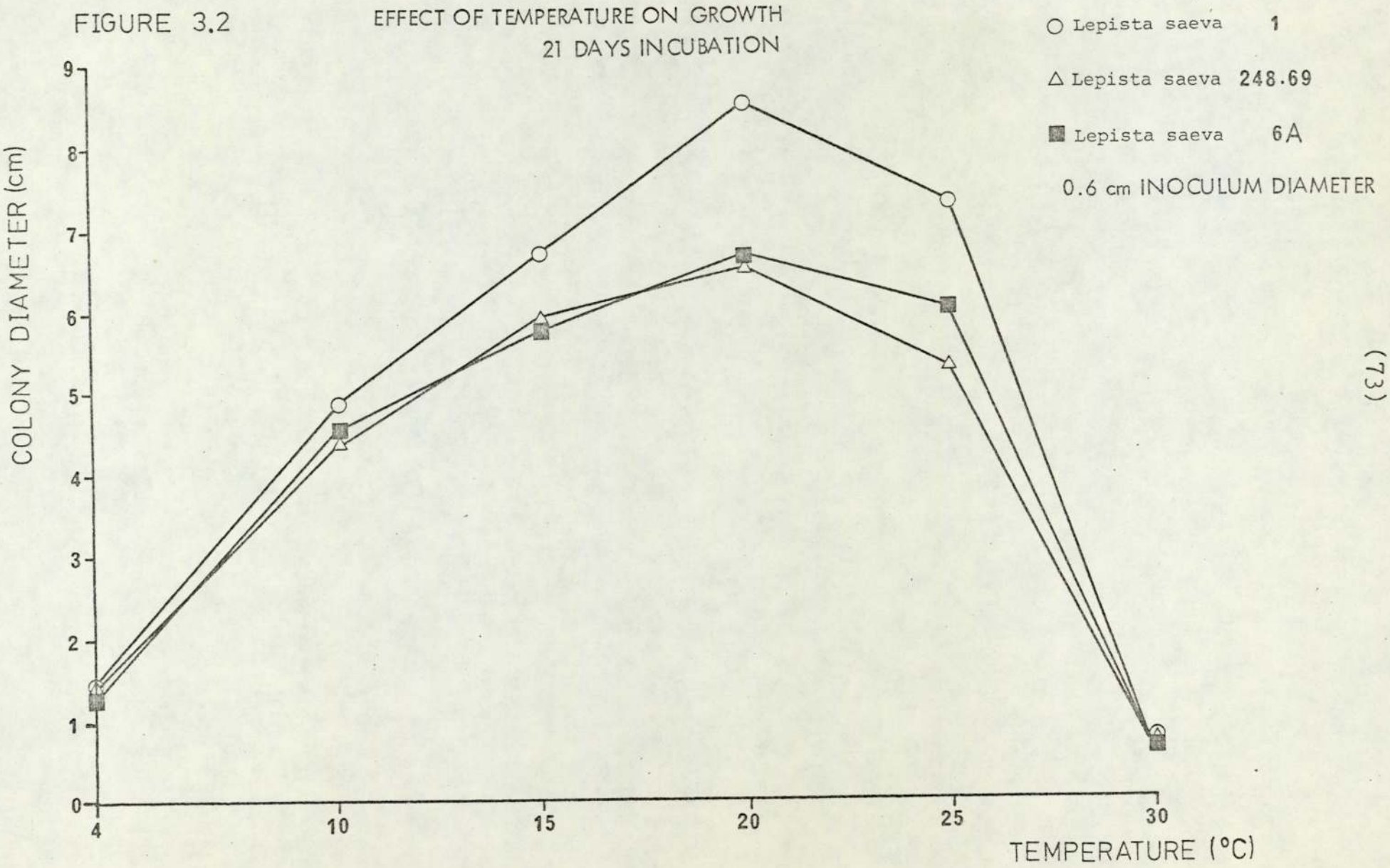
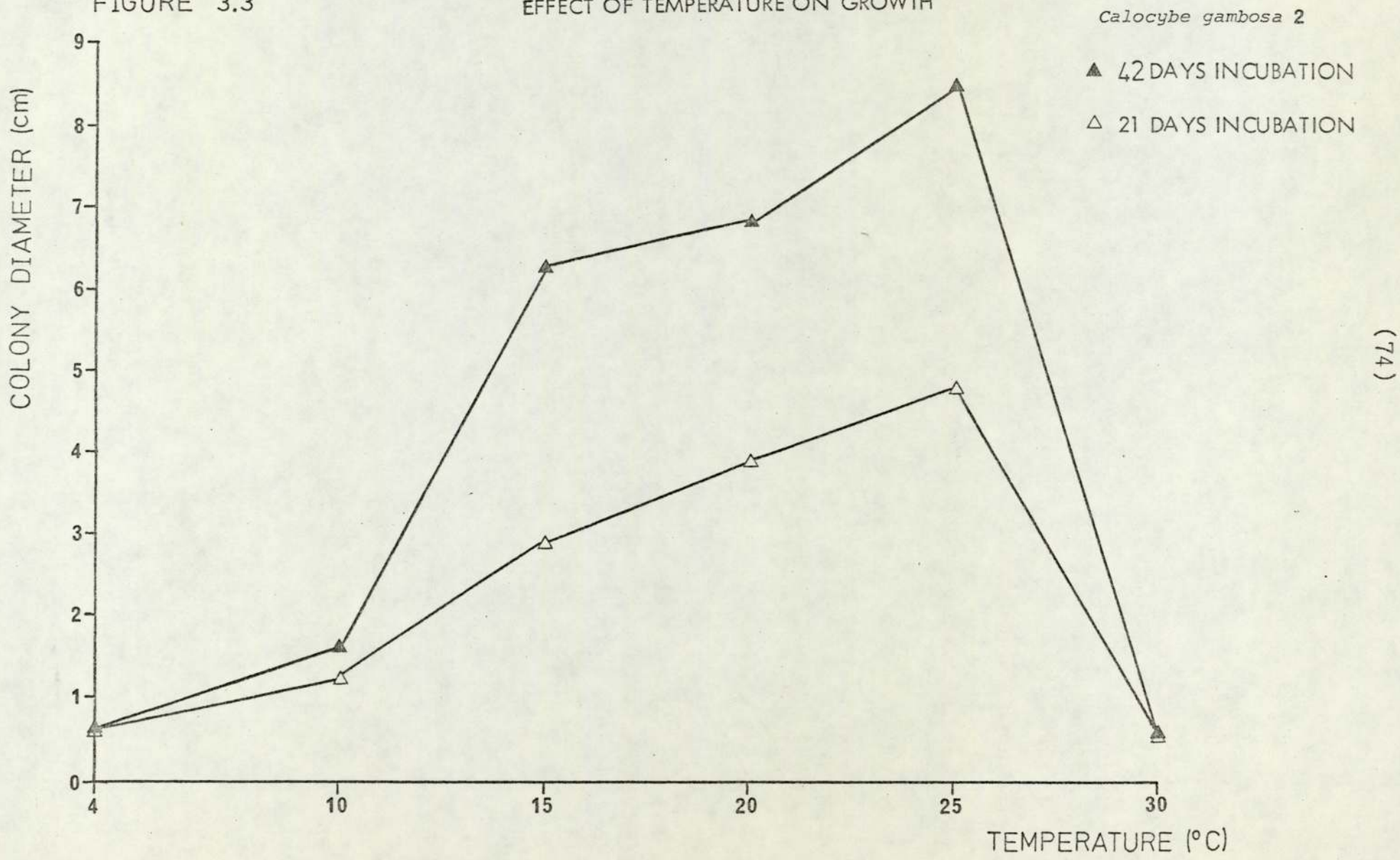

EFFECT OF TEMPERATURE ON GROWTH
21 DAYS INCUBATION
○ Lepista saeva 1
△ Lepista saeva 248.69
■ Lepista saeva 6A
0.6 cm INOCULUM DIAMETER
COLONY DIAMETER (cm)
0
1
2
3
4
5
6
7
8
9
4
10
15
20
25
30
TEMPERATURE (°C)

FIGURE 3.3

EFFECT OF TEMPERATURE ON GROWTH



of L. saeva isolates 1, 5, 6B and 7 and also to a lesser extent with isolate 248.69. Effects were autolysis of the mycelium with the agar medium turning dark brown, growth was difficult to resume from these colonies. Culture staling was not a feature of L. nuda isolates in this temperature range.

Growth of the C. gambosa isolate was optimal at 25°C with good growth at 15 and 20°C (Figure 3.3). Growth was markedly reduced at 10°C and inhibited at 4°C. No growth took place at 30°C.

SECTION 3

4:0 EFFECT OF ASH AND TRACE METALS ON GROWTH

Growth of the species L. nuda and L. saeva was very significantly greater ($P = 0.001$) on the complete medium than that attained on the basal salt solution (with the exception of L. nuda isolate 8) (Figures 4.1 and 4.2).

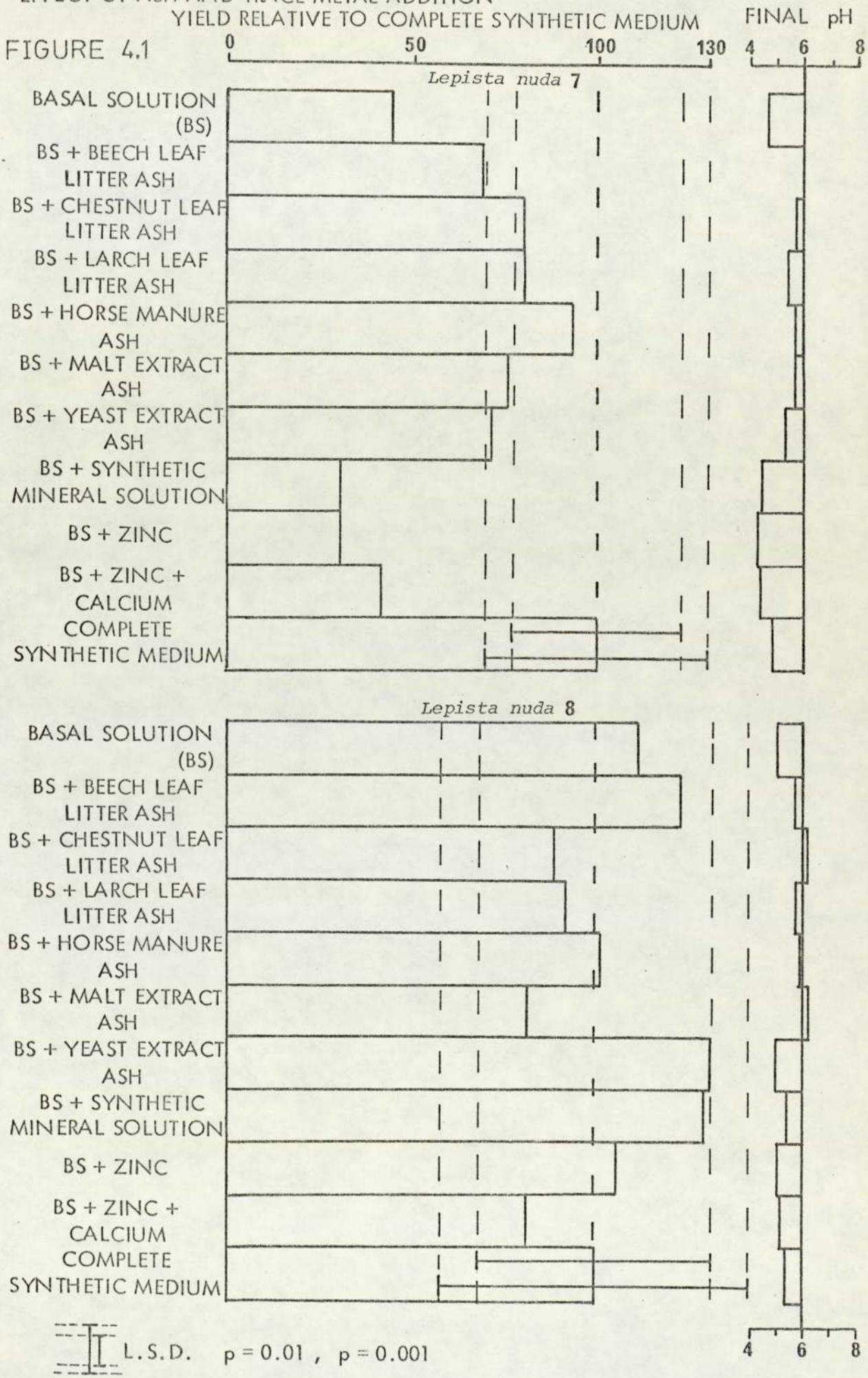
Components of the synthetic mineral solution, Ca, Mo, Cu, Mn and Zn did not significantly improve growth (exception L. saeva 6B), and did not equal the growth promotory effect of yeast and malt extract ash (exception L. nuda 8).

Zinc or zinc and calcium additions did not markedly affect growth, a slight increase in yield with calcium and zinc addition indicates that calcium is important, however, calcium exerts little effect at low concentrations (i.e. as a trace metal). The basal solution with zinc and calcium additions approximated the composition of the complete synthetic solution, with the exception of a greatly reduced concentration of calcium, therefore, the greatly improved yields in the latter are probably due to the ability of calcium to counteract the effects of high levels of metal ions antagonistic to growth or the build-up of inhibitory concentrations of intermediate metabolic products (possibly by chelation) such as certain of the Krebs cycle organic acids.

The beneficial effects of the addition of ash components

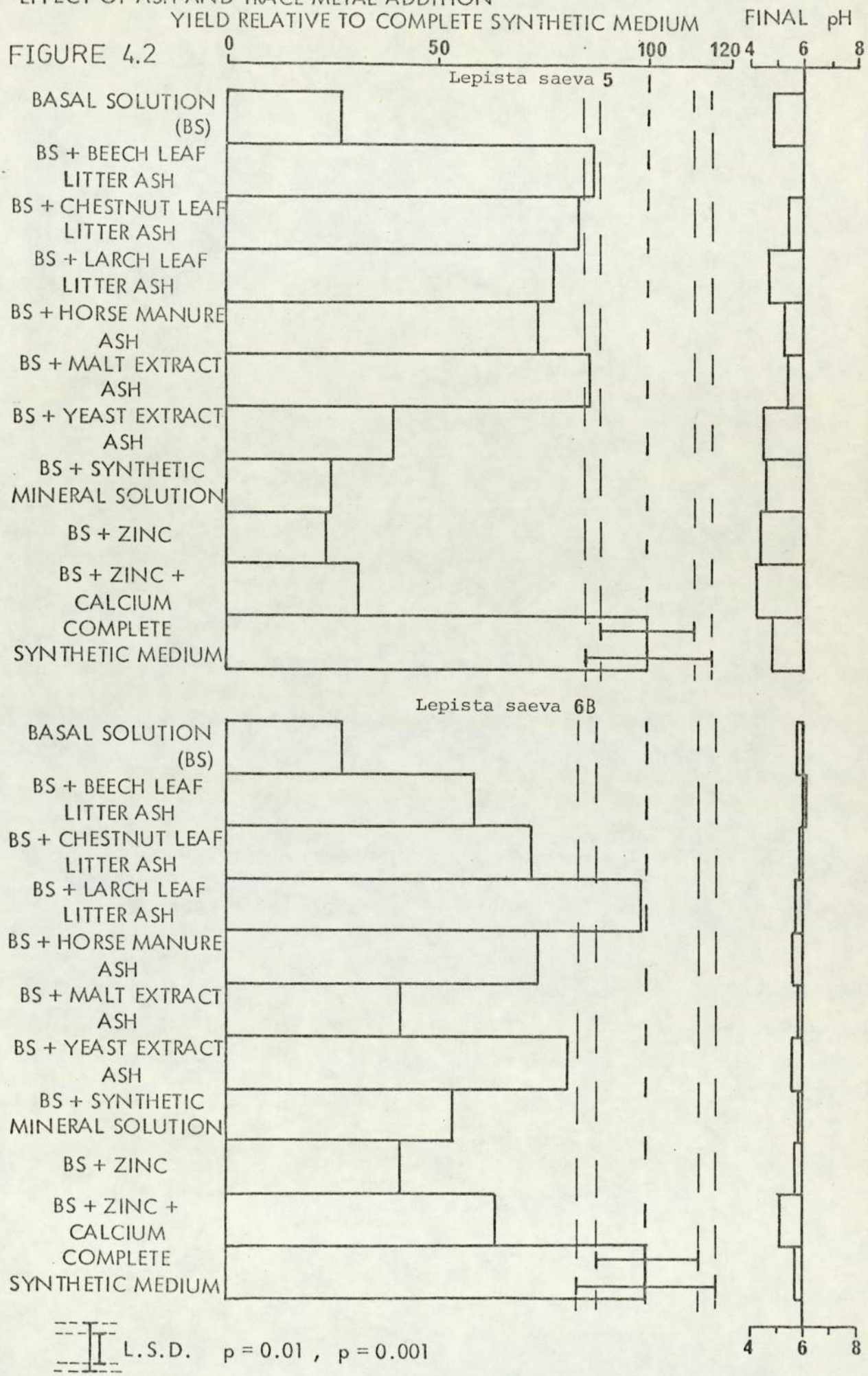
EFFECT OF ASH AND TRACE METAL ADDITION
YIELD RELATIVE TO COMPLETE SYNTHETIC MEDIUM

FIGURE 4.1



EFFECT OF ASH AND TRACE METAL ADDITION
YIELD RELATIVE TO COMPLETE SYNTHETIC MEDIUM

FIGURE 4.2



of leaves and manures was probably due to the calcium component or the addition of other metal ions particularly potassium and magnesium.

The exception-*L. nuda* isolate 8 — yielded higher on the basal solution than the complete medium. It is possible that metal ion components of the yeast extract and synthetic mineral solution were stimulatory to growth of this isolate.

5.0 EFFECT OF VITAMINS ON GROWTH

Growth on the complete vitamin solution minus pyridoxine thiamine, biotin or folic acid and with the vitamin-free control was significantly reduced ($P = 0.001$) within the species *L. nuda* and *L. saeva*, with the exception of *L. nuda* isolate 8 on media minus pyridoxine, biotin and folic acid, this isolate demonstrating a sole requirement for thiamine (Figures 5.1 and 5.2).

All isolates of these species demonstrated a clear requirement for thiamine, the requirement for biotin was only partial.

Absence of folic acid from the complete vitamin solution produced a growth reduction in both *L. saeva* isolates and in the *L. nuda* isolate 7, growth of the latter was also reduced when p-aminobenzoic acid (part of the folic acid moity) was deleted from the complete medium.

Growth of the *L. saeva* isolate 6B was signicantly reduced with removal of calcium pantothenate and niacin from the complete vitamin solution.

Low yields were produced by the *Calocybe gambosa* isolates (Figure 5.3). The cause may have been use of a purified asparagine preparation for the media, also possibly the additions lipoic acid, inositol or choline chloride may have been inhibitory to growth. It is possible that these substances caused a general suppression of yield by the *L. nuda* and *L. saeva* isolates.

Although the complete vitamin solution was formulated as a synthetic yeast extract vitamin source, actual addition of yeast extract had a clearly beneficial effect particularly in the case of the

FIGURE 5.1

VITAMIN REQUIREMENT FOR GROWTH

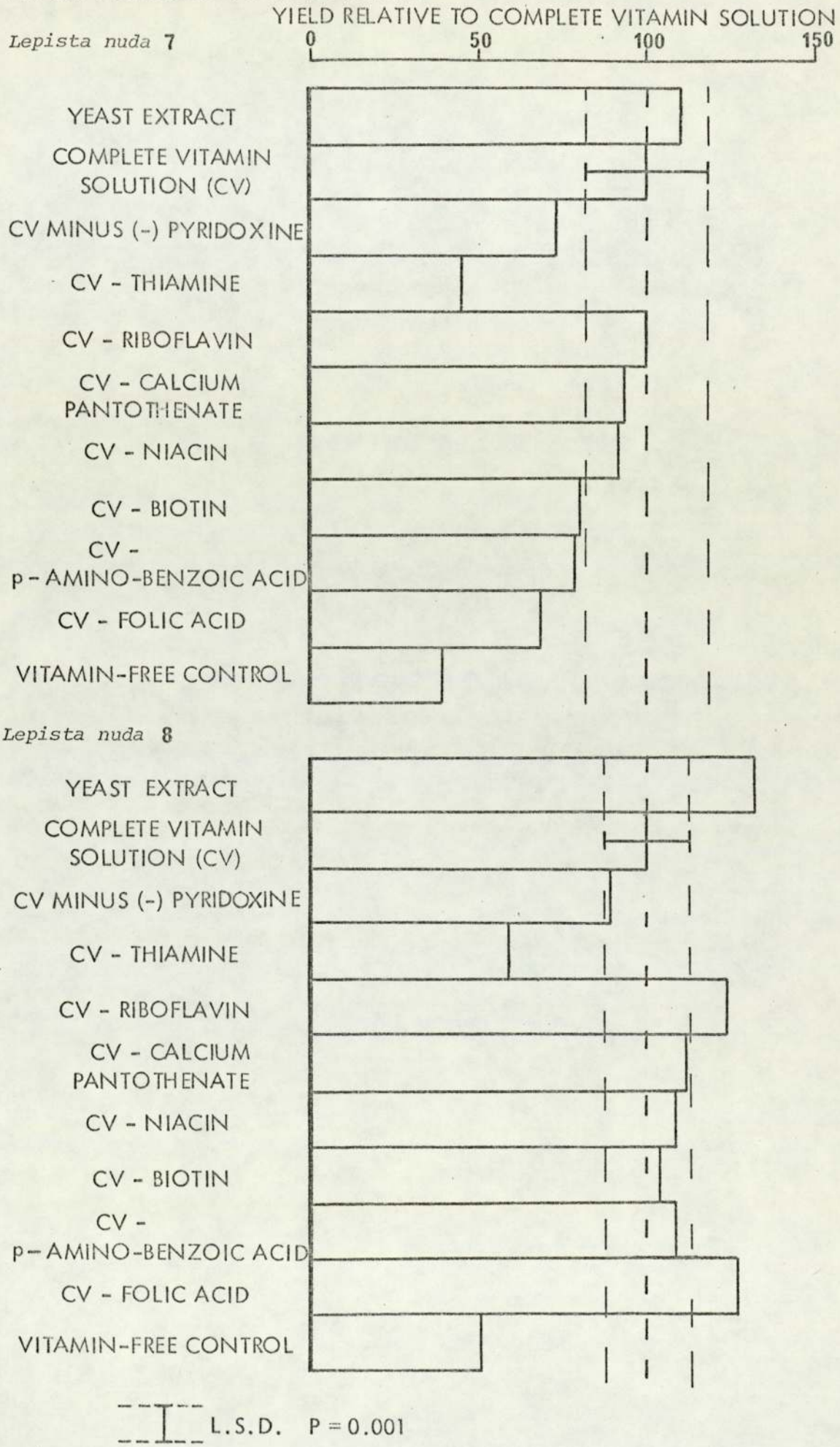


FIGURE 5.2
VITAMIN REQUIREMENT FOR GROWTH

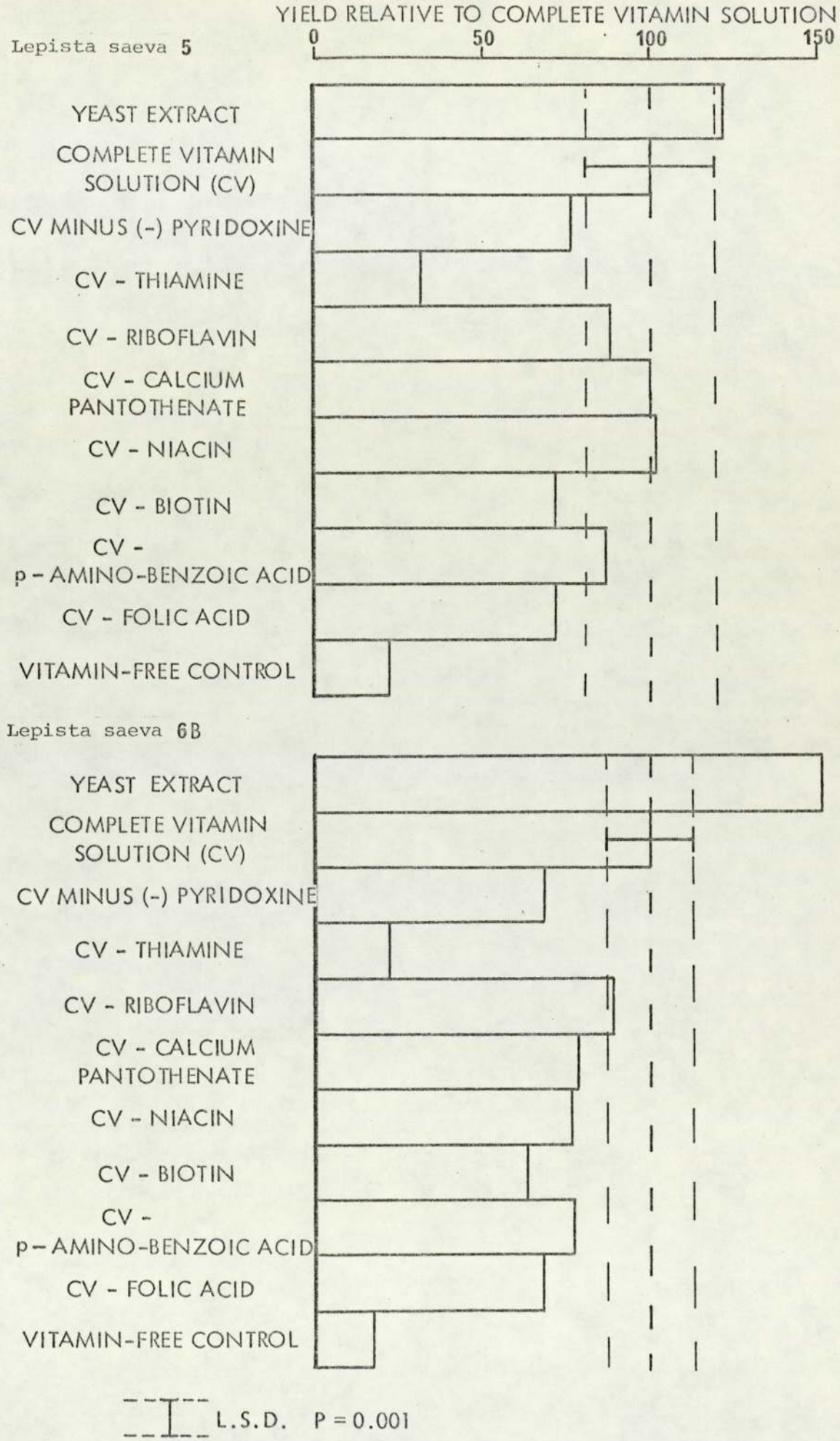
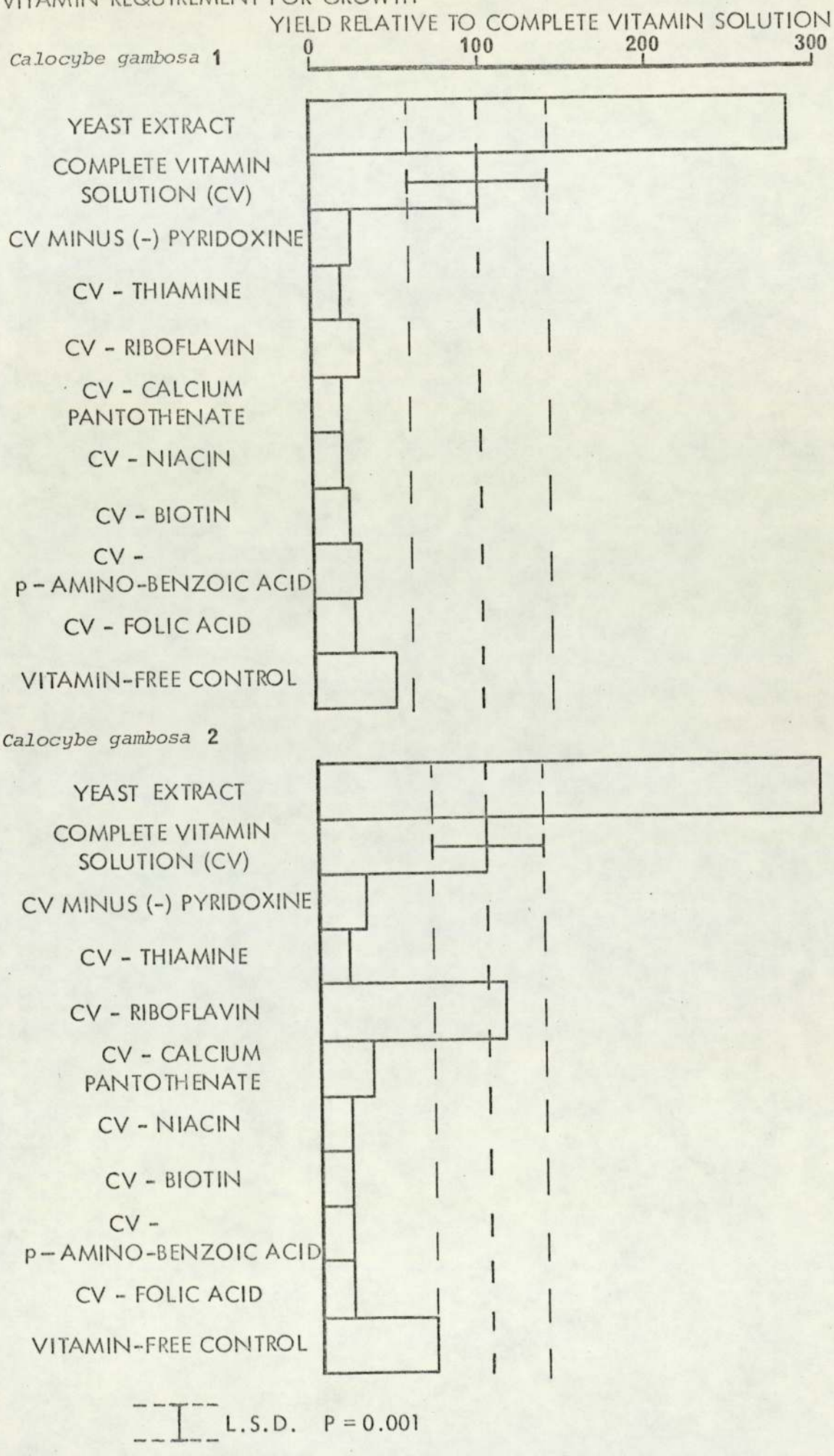


FIGURE 5.3
VITAMIN REQUIREMENT FOR GROWTH



C. gambosa isolates. This may have been due to the addition of amino-acids or other stimulatory substances contained within this source of yeast extract.

6:0 INFLUENCE OF CARBON : NITROGEN RATIO ON HYDROGEN ION CONCENTRATION AND YIELD

In an initial experiment with inadequately buffered media the final yields were influenced by the pH change in the media during growth (Figures 6.1 and 6.2.). At low C/N ratios (9:1) the hydrogen ion concentration shifted into the alkaline range, this was particularly marked in the case of the L. nuda isolate. With increase in sugar levels acids formed markedly reduced pH to low values, this change was strongest with increasing concentration of the carbon and nitrogen sources in the media.

In the second experiment yields were assessed at seven day intervals (together with the pH values of the pooled culture fluid) to 28 days. An increased level of phosphate buffering was employed, pH values did not alter markedly for either of the Lepista isolates, the greatest drop recorded was for the L. saeva isolate following 28 days incubation at 0.3% asparagine (0.5592 g%N) addition. (1.9 units) (Appendix Table 6.5-6)

Considering yields of the L. nuda isolate at 0.1 and 0.3 per cent additions of asparagine, (Figures 6.3 and 6.4) higher dry-weights were produced with increasing the C to N ratio. The best yield at 28 days for the 0.1% asparagine series was at a C/N ratio of 21:1.

A C/N ratio of 21:1 was also the most favourable ratio until 21 days incubation at the higher nitrogen level (0.5592g%N), at this point with nitrogen in excess a shift in the carbon level reduced the ratios of 25 and 28:1 to a more favourable balance enabling increased yields.

Yields of L. saeva for the 0.1864 g%N series (Figure 6.5) showed carbon limitation (assumption that mycelium contains

FIGURE 6.1

EFFECT OF CARBON : NITROGEN RATIO ON YIELD

GROWTH AT FOUR CONCENTRATIONS OF NITROGEN

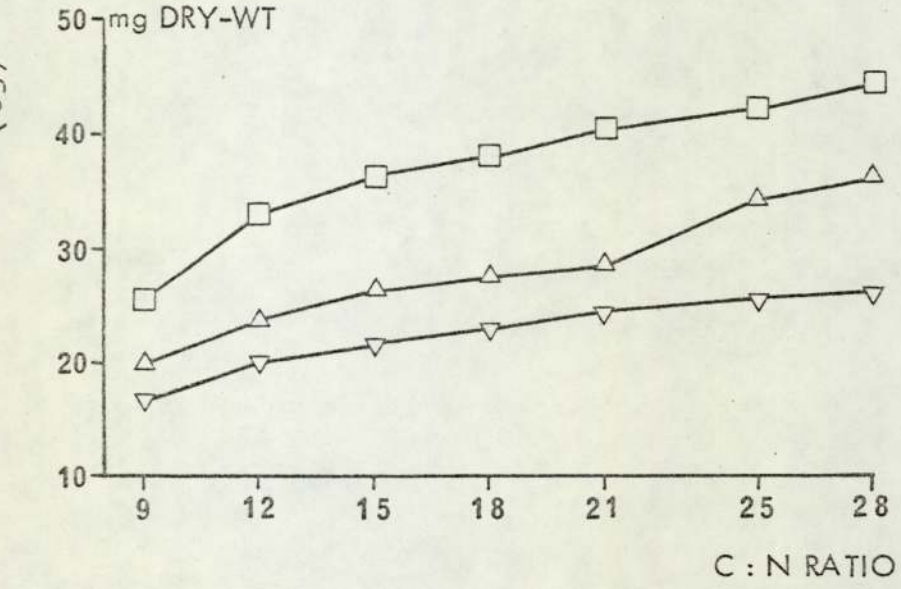
28 DAYS INCUBATION

KEY TO FIGURES 6.1 & 6.2

	PER CENT ASPARAGINE	NITROGEN CONCENTRATION (g/L)
□	0·3	0·56
△	0·2	0·372
◇	0·1	0·186
▽	0·05	0·093

Calocybe gambosa 2

YIELD



Calocybe gambosa 2

pH CULTURE FLUID

POST–INCUBATION

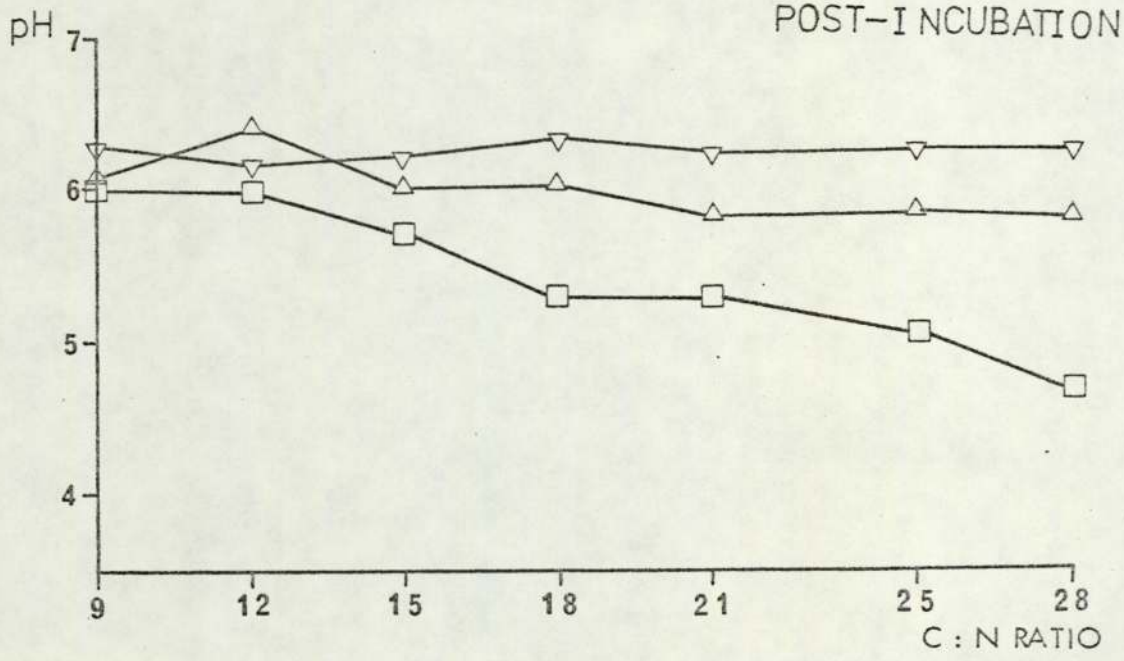
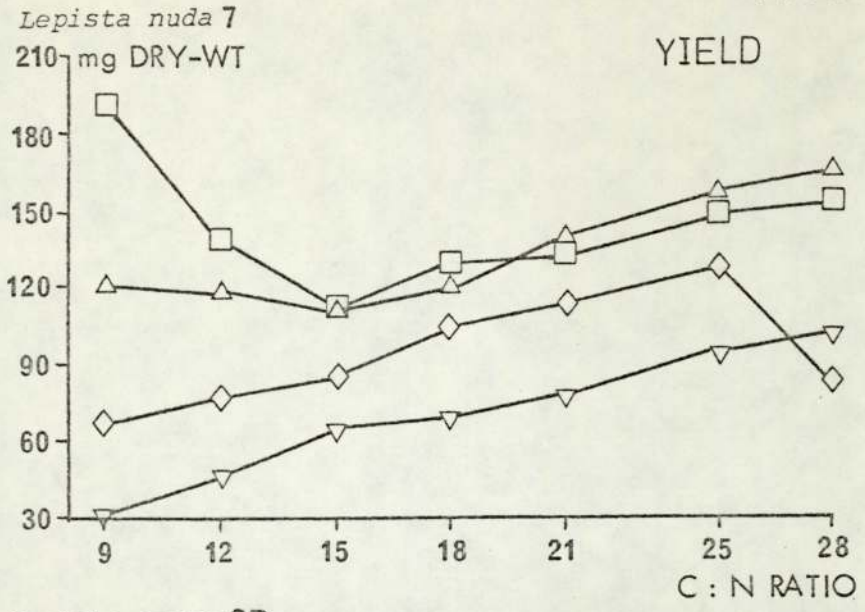
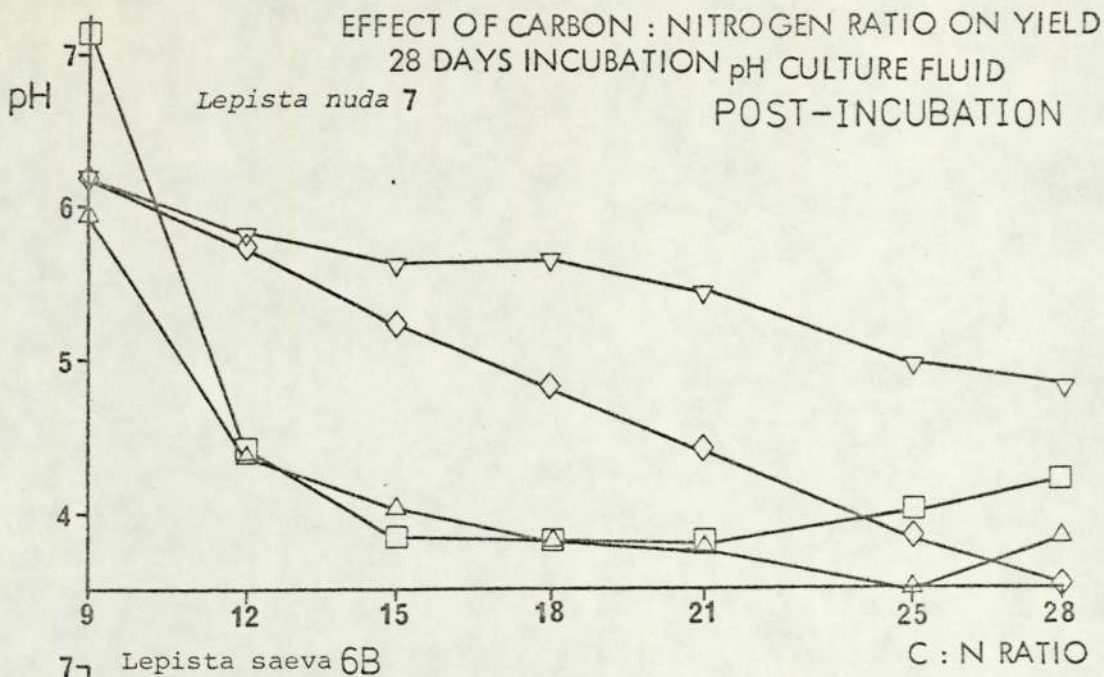
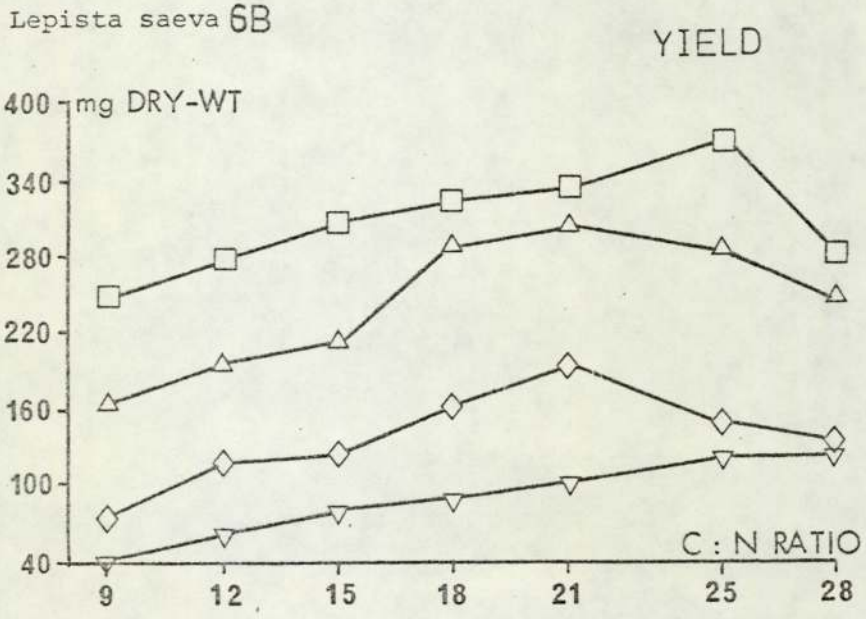
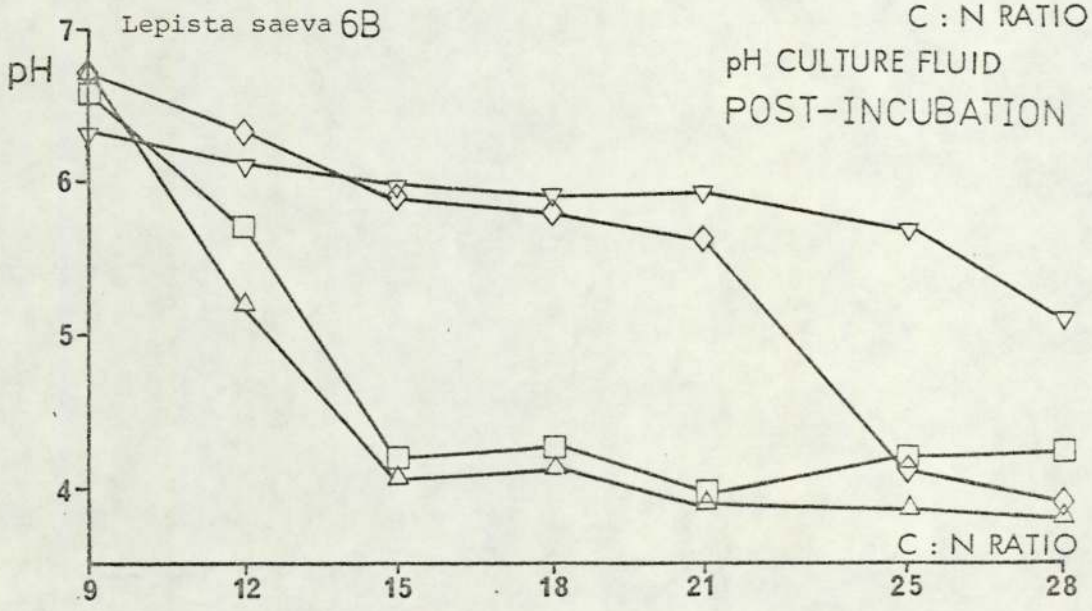


FIGURE 6.2


Lepista nuda 7
YIELD
mg DRY-WT
210
180
150
120
90
60
30
9
12
15
18
21
25
28
C : N RATIO


EFFECT OF CARBON : NITROGEN RATIO ON YIELD
28 DAYS INCUBATION pH CULTURE FLUID
POST-INCUBATION
Lepista nuda 7
pH
7
6
5
4
9
12
15
18
21
25
28
C : N RATIO


Lepista saeva 6B
YIELD
mg DRY-WT
400
340
280
220
160
100
40
9
12
15
18
21
25
28
C : N RATIO

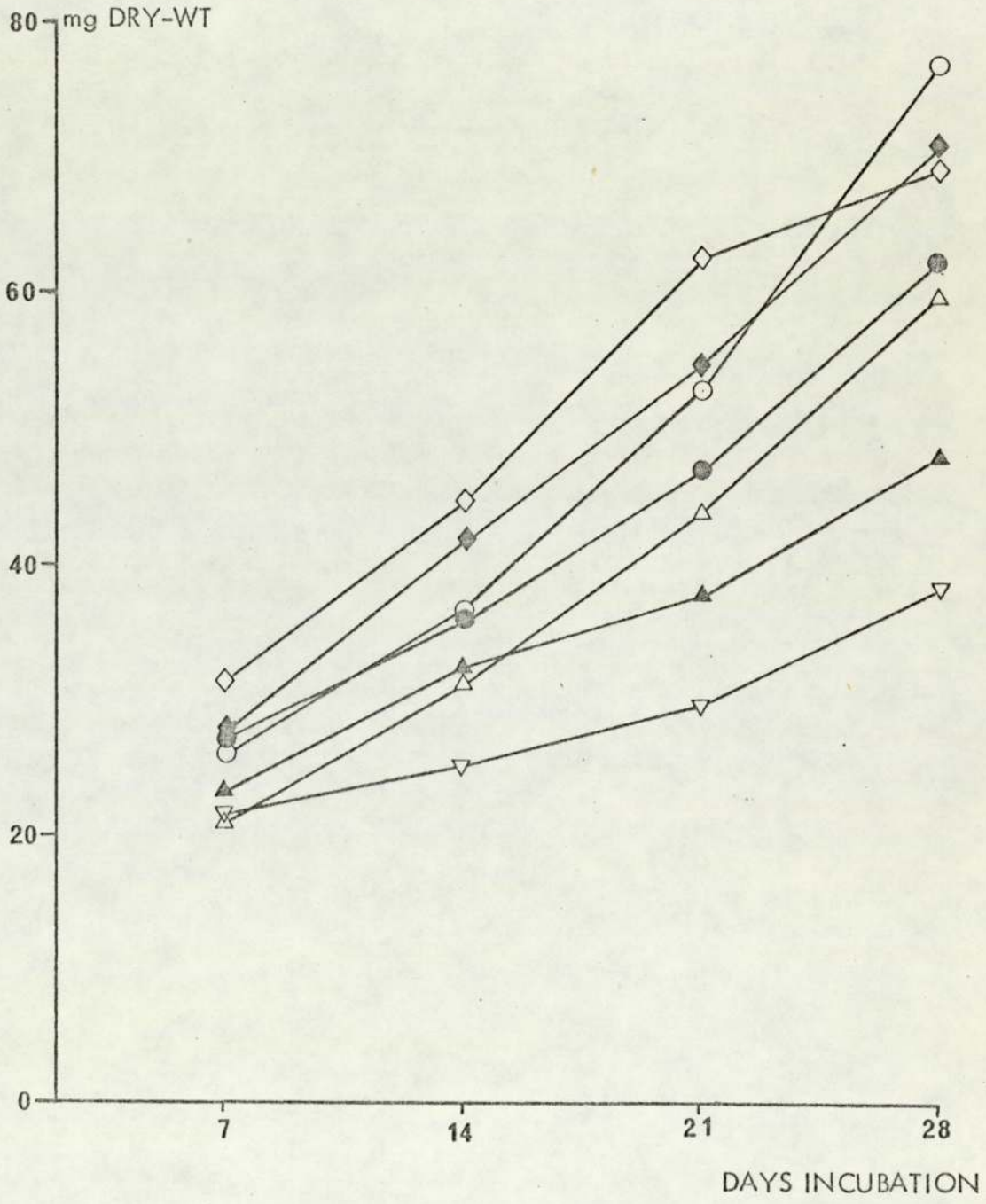

Lepista saeva 6B
pH CULTURE FLUID
POST-INCUBATION
pH
7
6
5
4
9
12
15
18
21
25
28
C : N RATIO

EFFECT OF CARBON : NITROGEN RATIO ON YIELD

FIGURE 6.3

0.186 g NITROGEN PER LITRE

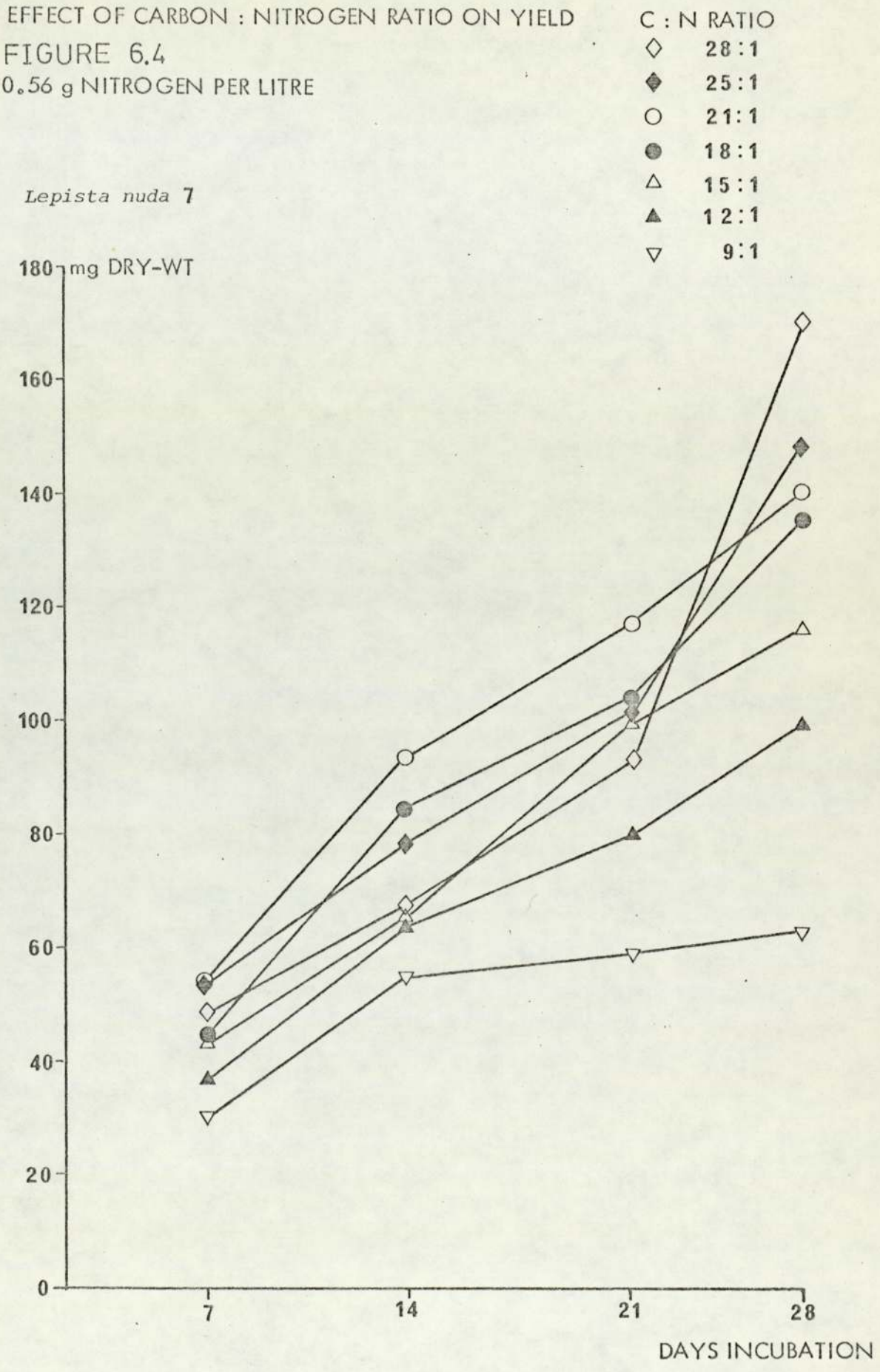
Lepista nuda 7



EFFECT OF CARBON : NITROGEN RATIO ON YIELD

FIGURE 6.4

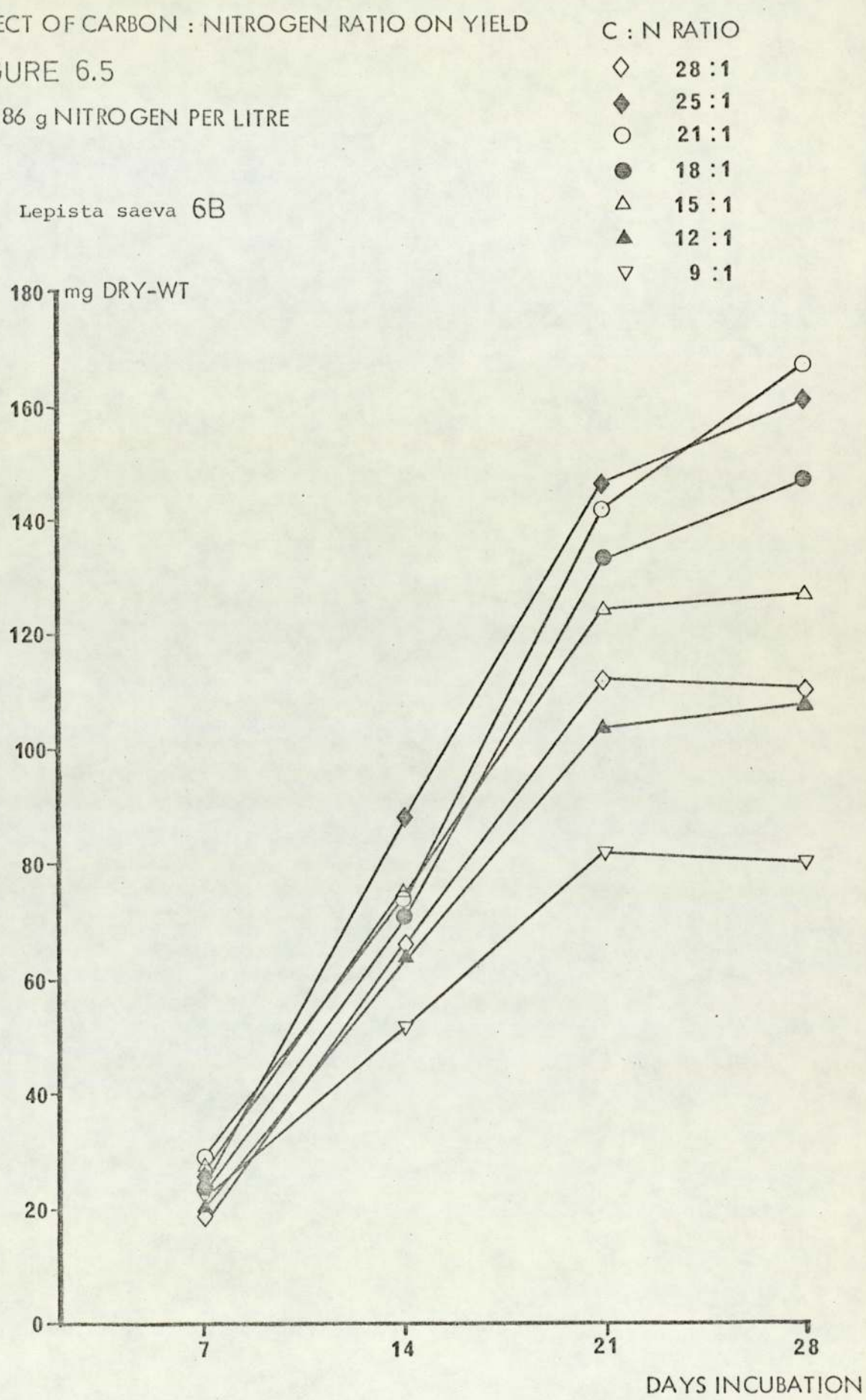
0.56 g NITROGEN PER LITRE



EFFECT OF CARBON : NITROGEN RATIO ON YIELD

FIGURE 6.5

0.186 g NITROGEN PER LITRE



EFFECT OF CARBON : NITROGEN RATIO ON YIELD

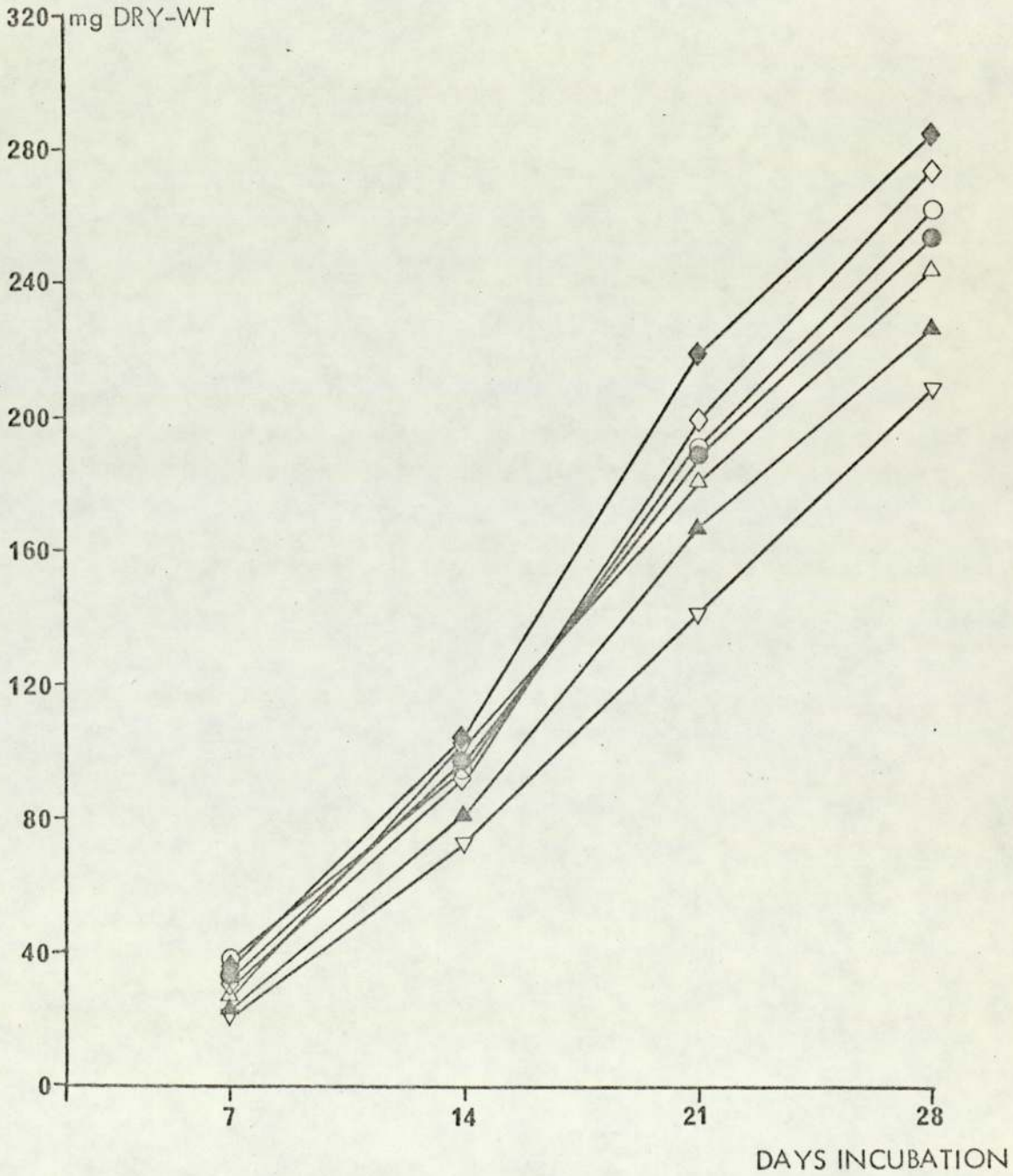
FIGURE 6.6

0.56 g NITROGEN PER LITRE

C : N RATIO

◇ 28 : 1
◆ 25 : 1
○ 21 : 1
● 18 : 1
△ 15 : 1
▲ 12 : 1
▽ 9 : 1

Lepista saeva 6B



c. 50% carbon) at 21 days, with concurrent reduction in yields at 28 days, also with nitrogen at 28 days becoming limiting (assumption of mycelial nitrogen content at c. 5%). The C/N ratio of 21:1 produced highest yields at 28 days.

For the 0.3% asparagine series (0.5592g %N) (Figure 6.6) the C/N ratio of 25:1 produced highest yields throughout. At the highest yields (284 mg) nitrogen started to become limiting. At the 9:1 ratio with the yields attained (208.6 mg) carbon level became limiting, thoughout the remaining ratios carbon and nitrogen were not limiting factors. Overall the L. saeva isolate demonstrated an ability to grow at a sustained high rate in media becoming increasingly deficient in carbon and nitrogen, including an ability to reduce its mycelial nitrogen content below that of 5%.

Average yields of both Lepista species were approximately doubled at 0.5592g %N compared to the 0.1864g %N series, these series represent two extremes, carbon limitation of the L. saeva isolate occurred in the 0.1864g %N series, together with some nitrogen limitation. In the 0.5592g %N series both carbon and nitrogen were generally present in excess, also pH shift in concentrated media at high C/N ratios is difficult to control, therefore, for the purposes of further studies the compromise of 0.3728g %N (2g Asparagine per litre) was chosen at a C/N ratio of c.22:1 (a glucose content of 20g per litre).

7:0 UTILISATION OF A RANGE OF SINGLE AND COMBINED CARBON SOURCES

The polysaccharide, tri - and disaccharides dextrin, raffinose, maltose, sucrose and trehalose produced higher yields than glucose with L. nuda isolate 7 (Figure 7.1). The polysaccharides glycogen, starch, inulin, the disaccharide cellobiose and the monosaccharides mannose and fructose also enabled high dry weights to be achieved.

Growth of the L. saeva isolate 6B was best on glucose (Figure 7.2). Mannose, dextrin, sucrose, fructose and starch, dry-weights decreasing respectively were excellent sources of carbon following glucose. The polysaccharides inulin, glycogen and xylan

FIGURE 7.1
UTILISATION OF CARBON SOURCES

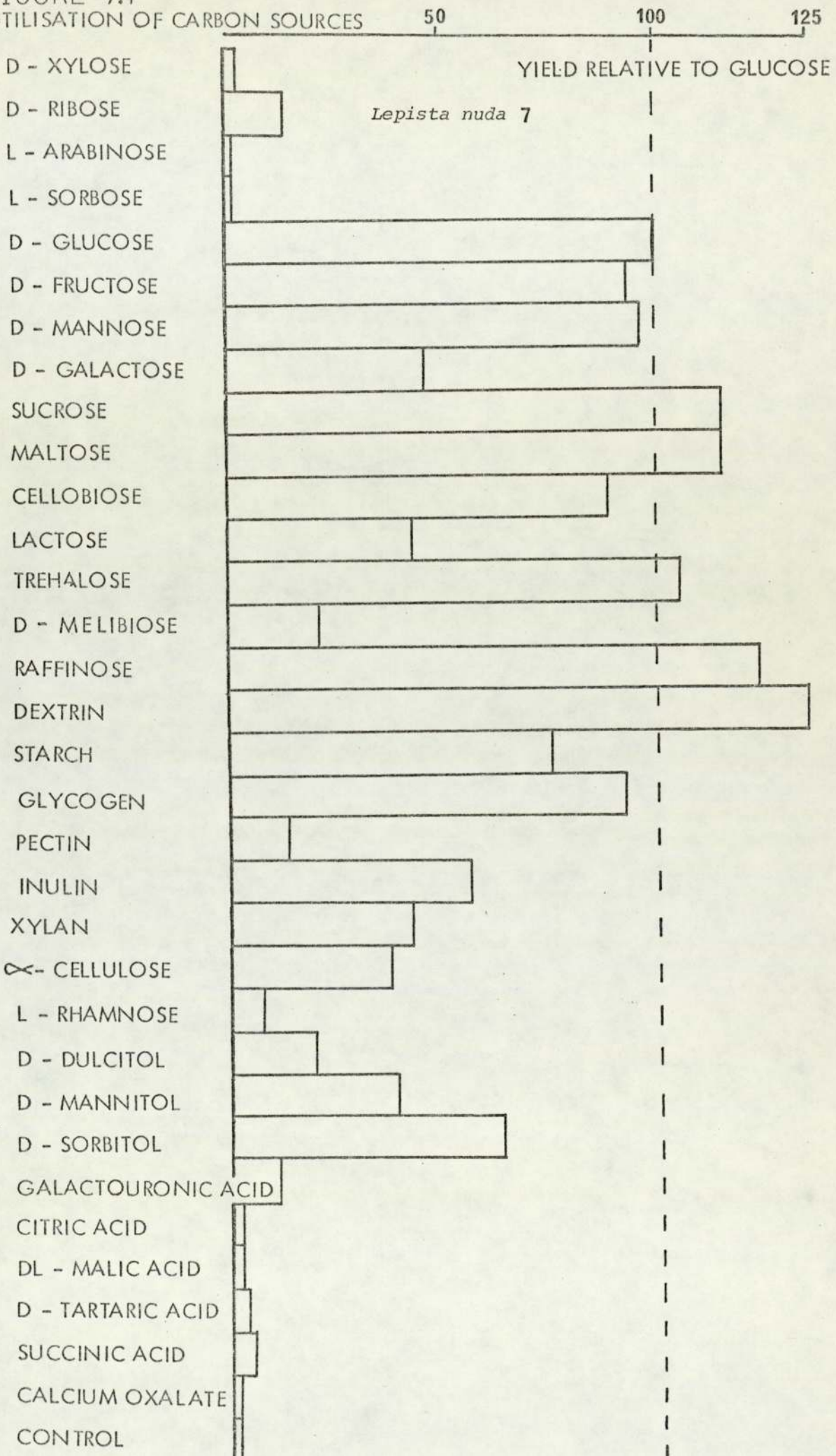


FIGURE 7.2

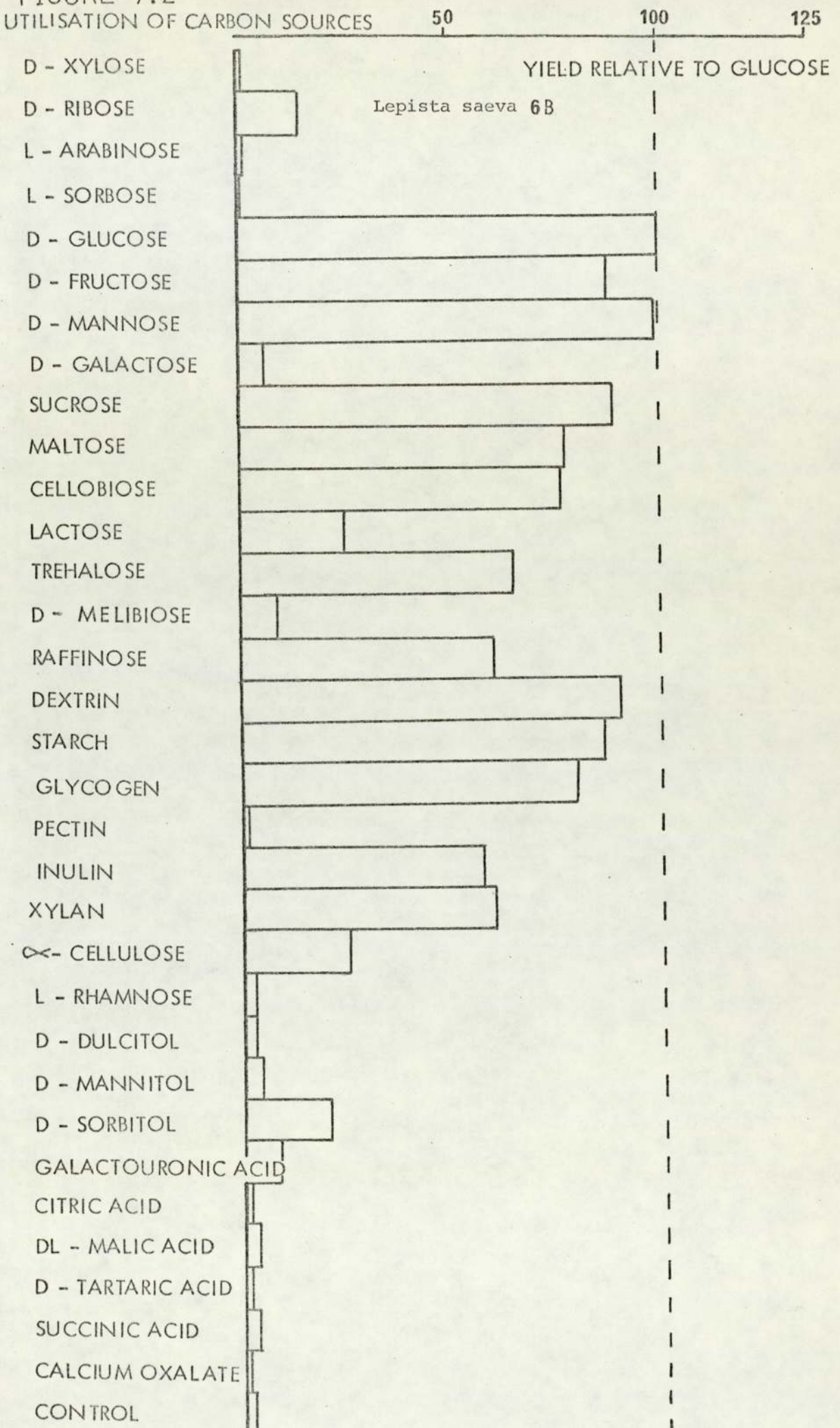

UTILISATION OF CARBON SOURCES
50
100
125
YIELD RELATIVE TO GLUCOSE
Lepista saeva 6B
D - XYLOSE
D - RIBOSE
L - ARABINOSE
L - SORBOSE
D - GLUCOSE
D - FRUCTOSE
D - MANNOSE
D - GALACTOSE
SUCROSE
MALTOSE
CELLOBIOSE
LACTOSE
TREHALOSE
D - MELIBIOSE
RAFFINOSE
DEXTRIN
STARCH
GLYCOGEN
PECTIN
INULIN
XYLAN
CELLULOSE
L - RHAMNOSE
D - DULCITOL
D - MANNITOL
D - SORBITOL
GALACTOURONIC ACID
CITRIC ACID
DL - MALIC ACID
D - TARTARIC ACID
SUCCINIC ACID
CALCIUM OXALATE
CONTROL

FIGURE 7.3

UTILISATION OF CARBON SOURCES

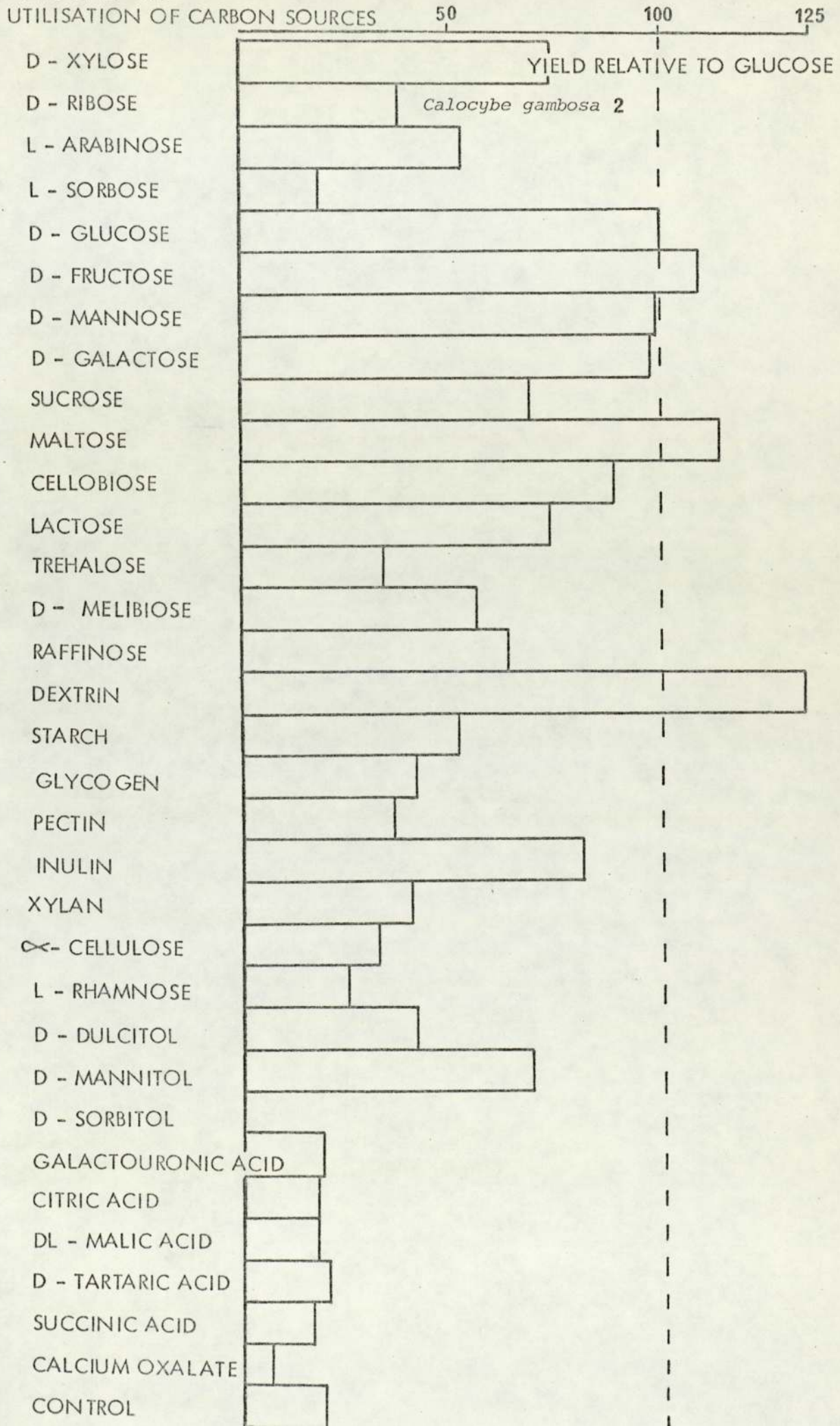


TABLE 7.1 GROWTH ON COMBINED CARBON SOURCES
(COMPARED WITH D-GLUCOSE)

CARBON SOURCE	Lepista nuda 7	Lepista saeva 6B	Calocybe gambosa 2
	mg	mg	mg
D - GLUCOSE	212.7	345.0	18.2
STARCH + MALTOSE + GLUCOSE	224.7	333.0	21.8
PECTIN + GALACTOSE + GLUCOSE	150.7	44.2	11.8
INULIN + FRUCTOSE + GLUCOSE	213.4	313.6	13.4
DEXTRIN + MALTOSE + GLUCOSE	230.2	275.7	20.0
MALTOSE + FRUCTOSE + GLUCOSE	252.9	308.5	18.2
SUCROSE + FRUCTOSE + GLUCOSE	195.1	358.7	16.2
GALACTOSE + FRUCTOSE + GLUCOSE	207.1	308.3	16.9
RIBOSE + ARABINOSE + XYLOSE	3.2	1.7	16.5
CELLOBIOSE + MALTOSE + SUCROSE	235.8		10.8
MANNOSE + SORBOSE + ARABINOSE	98.6	96.2	12.2
GLUCOSE + FRUCTOSE, 1,6 DIPHOSPHATE	193.5	277.2	17.0
CONTROL	3.6	4.4	3.3

were also high yielding carbon sources, the trisaccharide raffinose and the disaccharidetrehalose, a common fungal reserve carbohydrate, supported good yields.

Growth of L. saeva on pectin was negligible, poor growth was also demonstrated by L. nuda, both achieved a low yield with the monomer galactouronic acid as carbon source. Both isolates produced moderate to low yields with cellulose, cellobiose - the terminal disaccharide(β (1-4) linked glucose) cleavage product of cellulose - served as an excellent source of carbon for each isolate. Growth was respectively better with galactose than ribose and vice versa for the L. nuda and L. saeva isolates.

Both species produced moderate yields on the hexitols sorbitol, mannitol and dulcitol. The pentose sugars, xylose and arabinose, and the keto-hexose sugar, sorbose, allowed only slight growth. The organic acids, citric, malic, tartaric and succinic together with calcium oxalate were *very* poor sources of carbon for both species. As atypical carbon sources, the disaccharide lactose, enabled moderate to poor yields, the trisaccharide melibiose and the methyl pentose sugar L-rhamnose poor growth with both isolates.

Combined sources of carbon produced yields, in general in slight excess of the yields, calculable for the corresponding amounts of the individual sugars (Table 7.1). The combination of starch, maltose and glucose was an excellent source of carbon for both species. Combination of carbon sources did not, however, markedly improve pectin, ribose, arabinose, xylose or sorbose as sources of carbon for the maintainance of mycelial growth and respiration.

The Calocybe gambosa isolate produced best growth on dextrin, maltose, fructose, glucose and mannose as the five highest yielding carbon sources (Figure 7.3). Good growth was produced on ribose, arabinose and xylose in combination and xylose and arabinose singly. Galactose and cellobiose produced good dry weights, also mannitol served as a *very* favourable carbon source.

The combination of starch, maltose and glucose was

excellent as a combined carbon source, producing the highest yield for this isolate in the series.

The organic acids, succinate, tartrate, malate and citrate plus calcium oxalate did not enable any significant growth by this species.

8:0 UTILISATION OF A RANGE OF ORGANIC AND INORGANIC NITROGEN SOURCES

Highest yields for all isolates (exception L. nuda 7) were produced on casein hydrolysate (Figures 8.1, 8.2 and 8.3). Asparagine was the best single source of nitrogen for all isolates with the exception of C. gambosa strain 2, which developed only very poorly on this substrate.

Growth on peptone was generally good for all species this was an excellent source of nitrogen for L. nuda isolates (approx., two-thirds to three-quarters that attained on asparagine), however, this was not a favourable nitrogen source for L. saeva strains (less than half yield attained on asparagine) and only a fair to poor source of nitrogen for C. gambosa isolates. Ammonium bicarbonate and urea proved to be good sources of nitrogen in general, the exceptions were poor growth on both by C. gambosa strain 1 and low yields on the latter by L. saeva strain 6B.

Growth on ammonium, sodium and potassium nitrate was highly variable, lower yields on the former was probably due to a sharp drop in pH values to inhibitory levels following utilisation of the ammonium component. Sodium and potassium nitrates were very good sources nitrogen for L. nuda isolate 7 and L. saeva isolate 5, but only a fair nitrogen source for L. nuda isolate 8 and a poor nitrogen source for L. saeva 6B. C. gambosa strain 1 grew well on ammonium nitrate, moderately with potassium nitrate and poorly on sodium nitrate, these were all poor nitrogen sources for isolate 2. Sodium nitrite proved toxic for all species compared with the control. Propionamide produced variable yields, but was in general only a fair source of nitrogen.

FIGURE 8.1

UTILISATION OF ORGANIC, AMMONIUM, NITRATE AND NITRITE NITROGEN

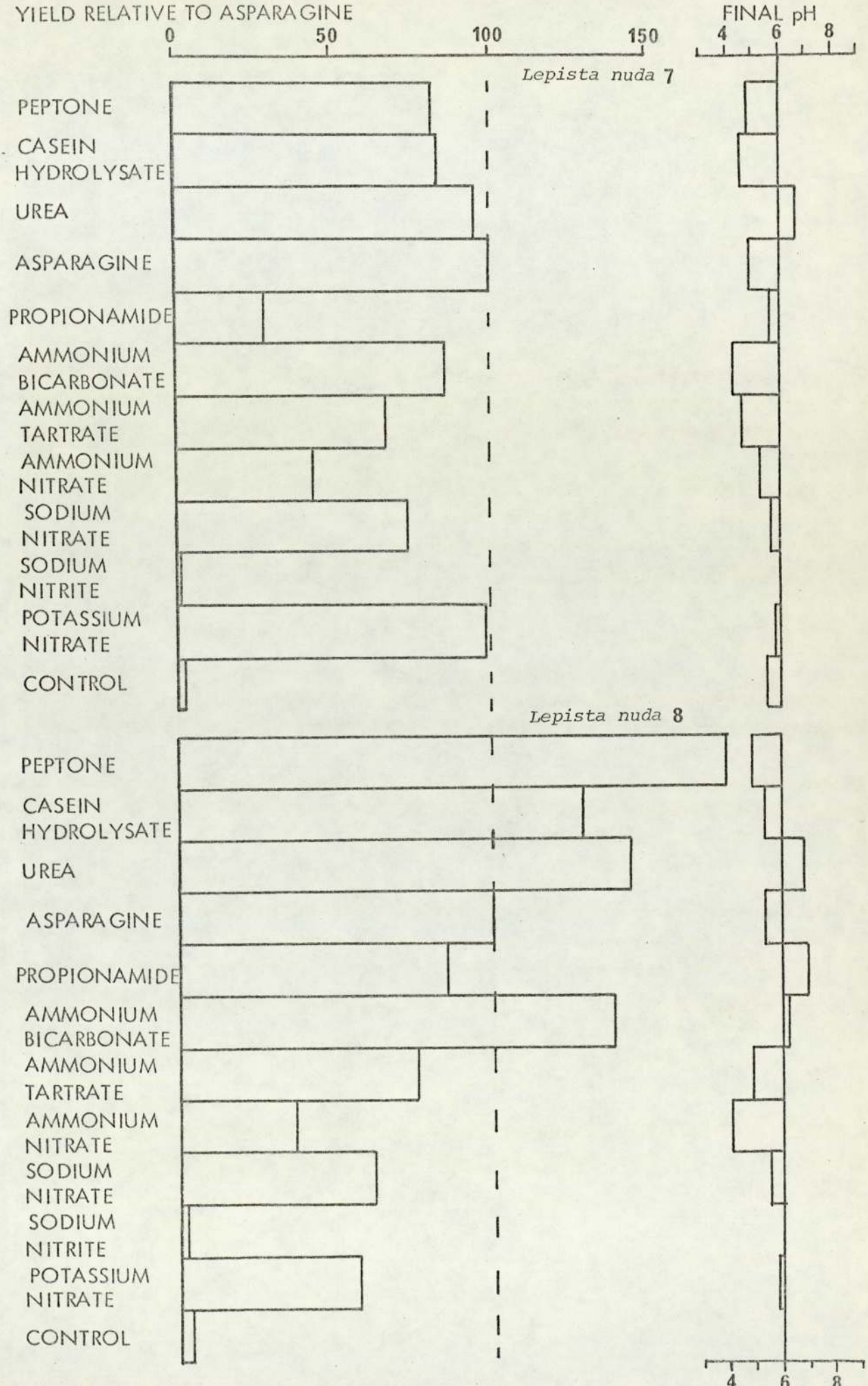


FIGURE 8.2

UTILISATION OF ORGANIC, AMMONIUM, NITRATE AND NITRITE NITROGEN

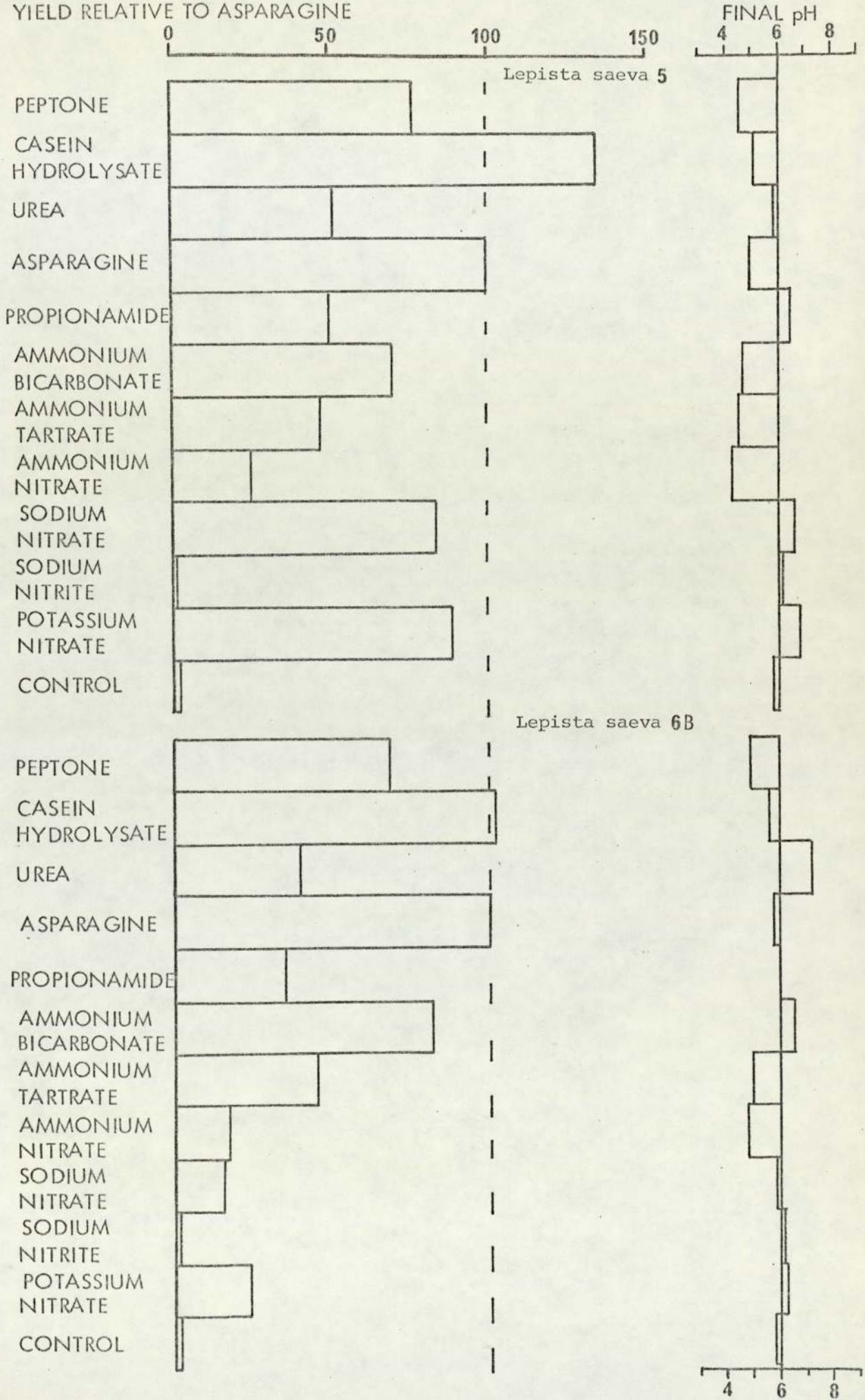
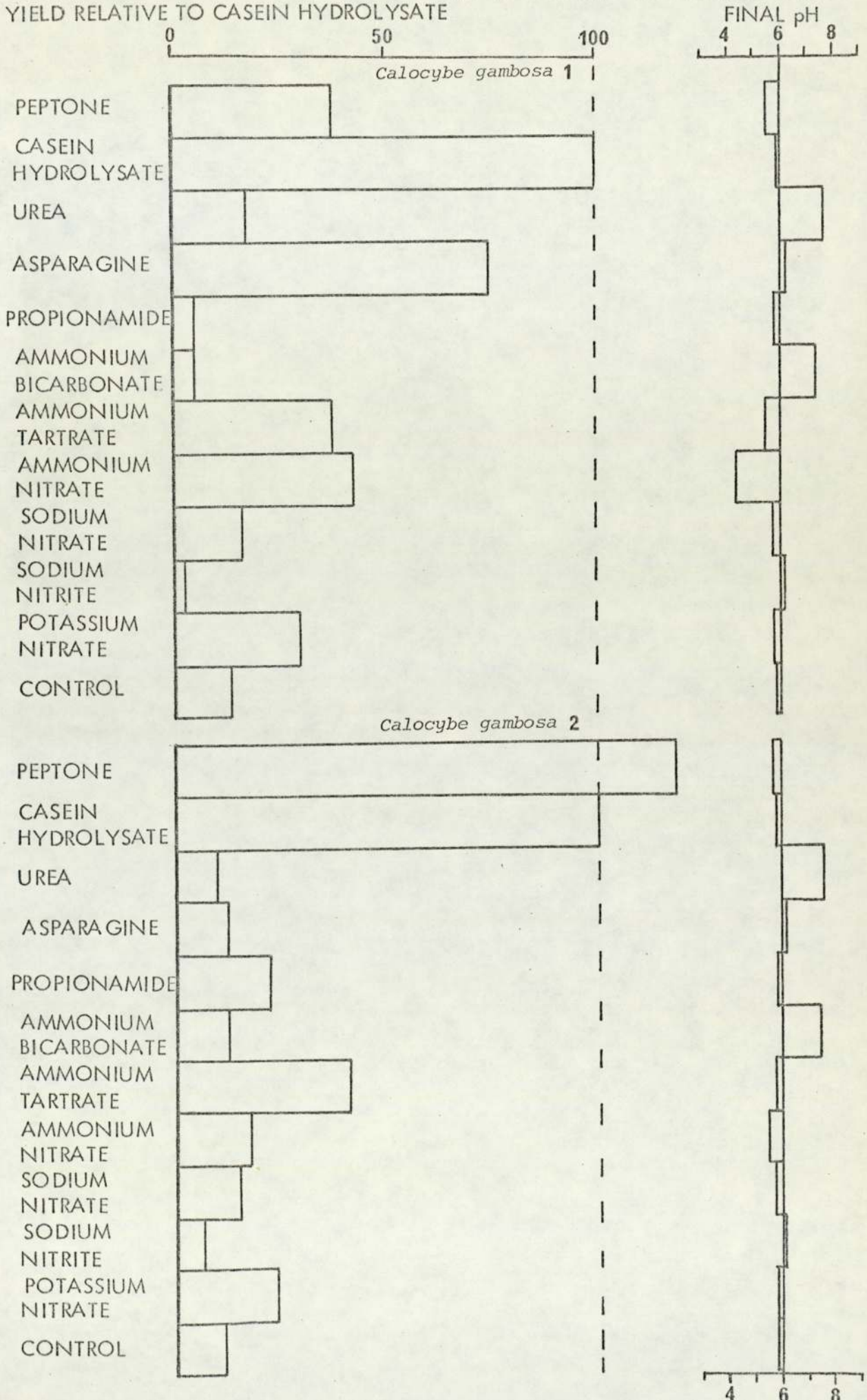


FIGURE 8.3

UTILISATION OF ORGANIC, AMMONIUM, NITRATE AND NITRITE NITROGEN



Change in the hydrogen ion concentration with metabolism of these substrates affected yields where this exceeded the buffer capacity. The most marked reduction in pH values occurred with ammonium nitrate and ammonium tartrate. A shift to higher pH values was produced by growth of L. saeva isolate 5 on potassium and sodium nitrates and also with isolate 6B on the former.

Growth on urea produced alkaline culture fluids following incubation, this was particularly marked in the case of the C. gambosa isolates, a shift to higher pH values also occurred with this species on ammonium bicarbonate and asparagine. This pH trend may reflect the net release of NH_3 into the culture fluid by metabolism of these substrates, possibly having a direct staling effect which rapidly inhibited further growth.

Propionamide and ammonium bicarbonate also caused a shift to higher pH values, than the control, in the culture fluids of the individual isolates of L. nuda and L. saeva 6B, which may reflect liberation of ammonia following metabolism of these chemicals.

8:1 Utilisation of a Range of Amino-acids as Single and Combined Sources of Nitrogen

Aspartic acid, leucine, glycine, alanine, glutamic acid, serine and arginine were, in order of declining yield, the best amino-acids as sole nitrogen sources for growth of the L. nuda isolate (7) (Figure 8.4). Aspartic acid and leucine produced favourable yields, both the amino-acid mix and casein hydrolysate were very good compound sources of nitrogen. Fair yields, approximately half that attained with asparagine were produced with threonine and methionine.

The L. saeva strain(6B) produced highest dry-weights declining respectively, with serine, glycine, glutamic acid, aspartic acid and alanine (Figures 8.5). The amide glutamine was only a poor source of nitrogen. Leucine and arginine supported low yields. Casein hydrolysate was an excellent compound nitrogen source, however, the amino-acid mix gave only low yields indicating an inhibitory influence

FIGURE 8.4
UTILISATION OF AMINO-ACID AND AMIDE NITROGEN SOURCES

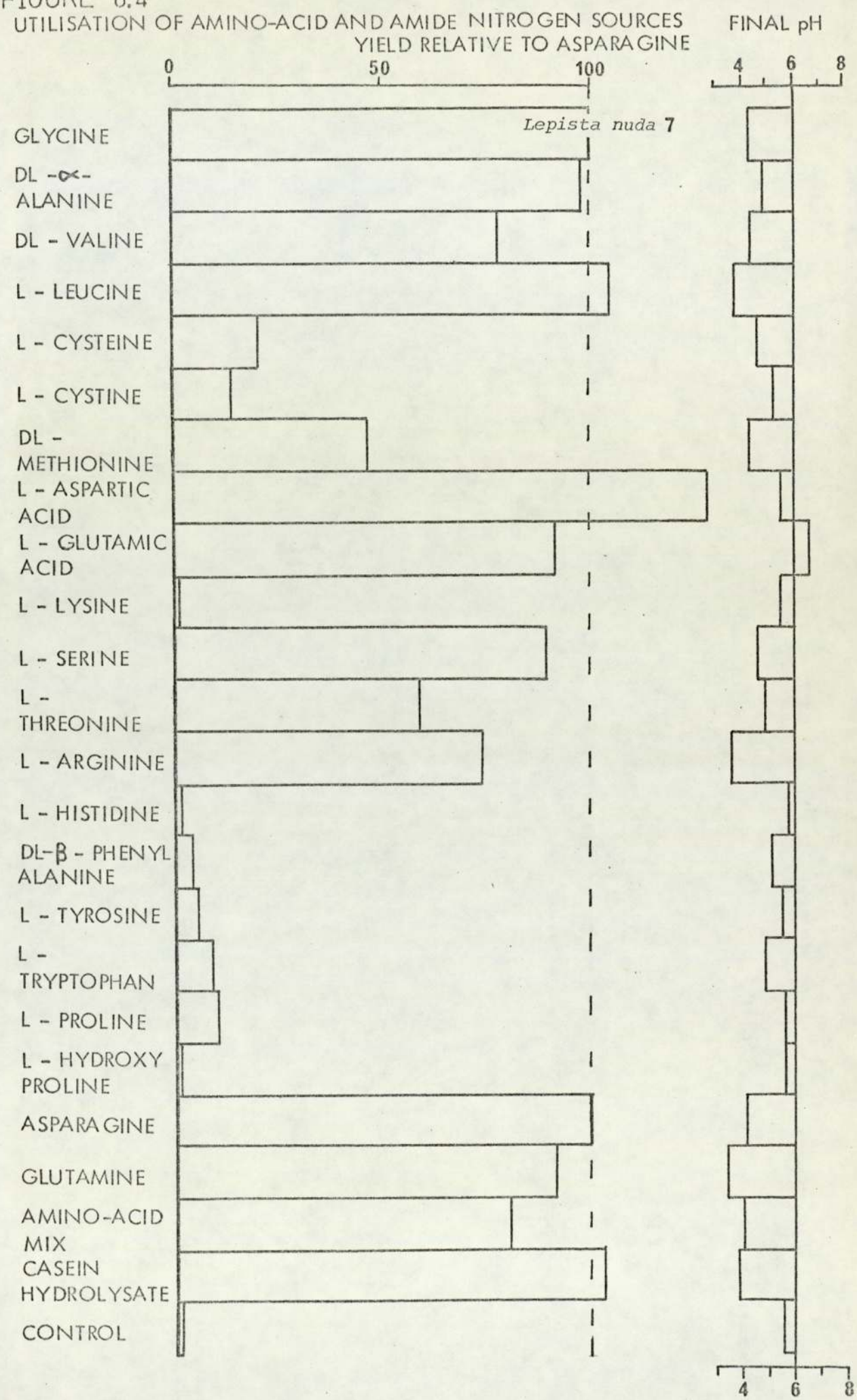


FIGURE 8.5
UTILISATION OF AMINO-ACID AND AMIDE NITROGEN SOURCES

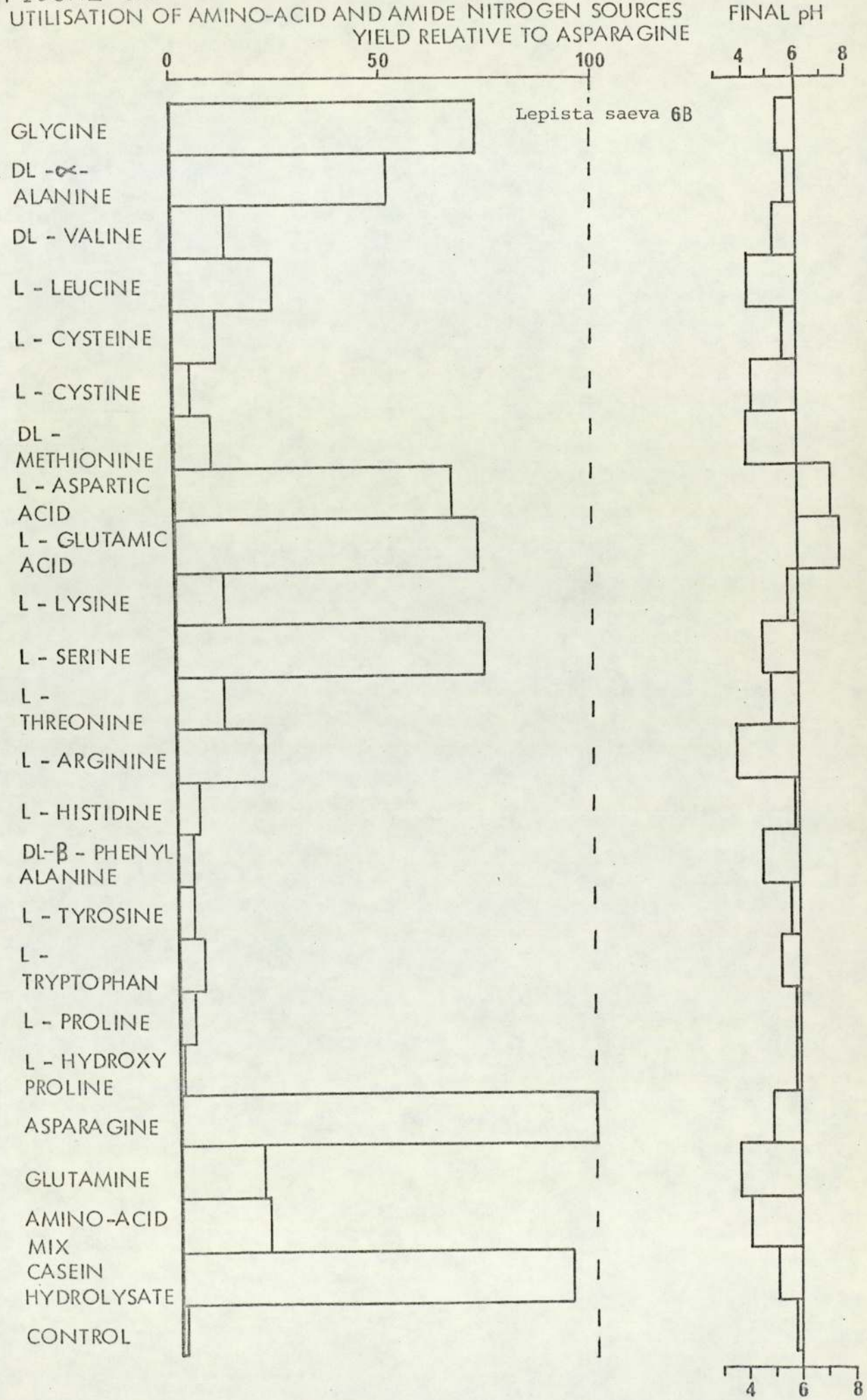


FIGURE 8.6

UTILISATION OF AMINO-ACID AND AMIDE NITROGEN SOURCES

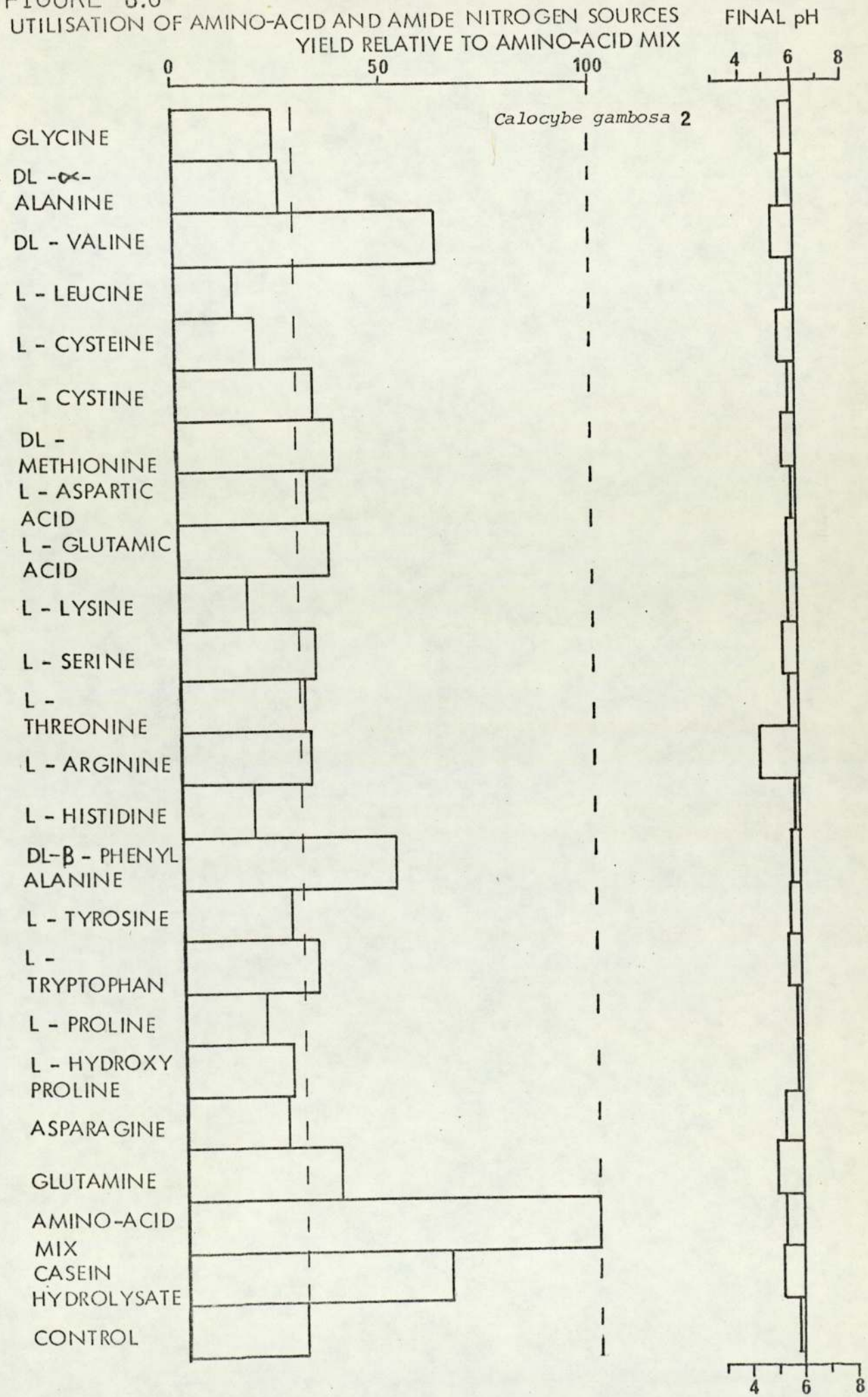


TABLE 8.1

VALUES FOR RESIDUAL GLUCOSE FOLLOWING INCUBATION OF L. SAEVA 6B ON SIX SOURCES OF AMINO-ACID NITROGEN.

NITROGEN SOURCES	RESIDUAL GLUCOSE (mg)
INITIAL	500
GLYCINE	135
SERINE	148
ASPARAGINE	42.5
GLUTAMINE	308.2
AMINO-ACID MIX	280
CASEIN HYDROLYSATE	82.5

Results determined for a single flask averaged from the pooled culture fluids of 3 replicate flasks by the 3, 5, dinitrosalcyclic acid method (Lindsay 1973).

possibly due to larger amounts of certain inhibitory amino-acids in this combination.

Good growth of the C. gambosa strain was produced with the amino-acid mix, less satisfactory growth on casein hydrolysate (Figure 8.6). The single amino-acids valine and phenylalanine produced highest yields. Methionine and glutamic acid plus its amide glutamine produced yields in slight excess of that given by the control.

Growth of L. nuda and L. saeva isolates showed a marked drop in the pH of the culture fluids following growth on arginine, leucine and methionine plus glutamine and the amino-acid mix, a slightly less marked shift in pH occurred following growth on glycine, valine, phenylalanine and cysteine.

Aspartic and glutamic acid produced net alkaline conditions for the L. saeva isolate, the latter caused a shift to neutrality with the L. nuda strain. Growth on casein hydrolysate produced a significant pH drop in the culture fluid of the L. saeva isolate.

The C. gambosa isolate produced a marked change to acid values in the culture fluid following growth on arginine.

The culture fluid of the L. saeva isolate was dark black following growth on tyrosine and the amino-acid mix, and brown with growth on casein hydrolysate, a black colouration of the medium was produced by the L. nuda isolate on tyrosine, the C. gambosa isolate produced black submerged myceliumwith tyrosine, indicating release of melanin pigments or intermediates.

A dark yellow culture fluid was produced by the L. saeva isolate on glycine, arginine and asparagine and a pink colouration on proline. L. nuda (7) also produced a dark yellow discoloured medium on arginine.

The culture fluid of the C. gambosa isolate was bright orange-yellow except following growth on cysteine, cystine, lysine, histidine, phenylalanine, tyrosine, proline and hydroxyproline.

The L. nuda and L. saeva isolates produced significant amounts of a polysaccharide type material on the underside of the mycelial mat following growth on certain of the amino-acids. Accumulation of this material was heaviest following growth on glutamine. Medium production occurred on arginine with both species and singly by the L. nuda isolate on glycine, valine, cystine, with light accumulation by this species on alanine, glutamic acid, amino-acid mix and casein hydrolysate as sources of nitrogen.

Values for residual glucose following growth of the L. saeva isolate (Table 8.1) which attained a highest yield of 335.6 mg on asparagine indicate approximately nine-tenths utilisation of substrate glucose; utilisation of glucose was roughly proportional to final dry-weight.

The figures indicate that the glucose asparagine basal medium adapted in these studies was capable of supporting high yields and did not become at twenty-eight days incubation carbon or nitrogen limited.

SECTION 4

9:0 AFFECT OF ETHANOL, SIMPLE ORGANIC ACIDS, AND FATTY ACIDS AS MEDIUM SUPPLEMENTS

Both L. nuda and L. saeva isolates did not show significant ($P = 0.05$) growth promotion with these medium supplements (Table 9.1). The optimum concentration for addition of the organic acids was critical. Concentrations in excess of 100 mg/L strongly inhibited growth, additions of these chemicals at higher levels made pH difficult to control. A fall in yield was also marked with the higher levels of addition of oleic, linoleic and palmitic (L. nuda isolate) acids where growth was assessed in stationary liquid culture.

Acetate, oleic, linoleic, stearic and palmitic acids caused a considerable increase in density of colony growth, clearly

TABLE 9.1

ADDITION (ORGANIC ACID, ALDEHYDE, ALCOHOL)	CONCENTRATION mg/L	LEPISTA NUDA 7 mg	LEPISTA SAEVA 6B mg	CALOCYBE GAMBOSA 2 mg
Acetate (Acetic Acid)	50	144.2	189.6	7.2
	100	135.6	189.9	19.5
Acetaldehyde	50	132.6	198.2	6.5
	100	124.3	194.0	6.1
N-Propionic Acid	50	146.0	186.5	26.0
	100	151.4	176.7	9.0
Iso-Valeric Acid	50	128.6	198.4	31.7
	100	151.1	176.5	25.7
N-Butyric Acid	50	131.3	171.8	62.7
	100	137.3	172.2	12.9
Ethanol	100	125.7	198.9	10.0
	500	126.0	201.9	10.6
Oleic Acid (18:1)	100	153.1	178.0	16.6
	500	85.9	86.0	13.0
Linoleic Acid (18:2)	100	137.2	142.3	15.9
	500	72.4	49.9	4.2
Palmitic Acid (16:0)	100	158.0	154.6	12.6
	500	92.0	154.9	12.0
Stearic Acid (18:0)	100	149.3	195.6	8.7
	500	108.0	193.8	6.5
CONTROL		146.3	190.3	14.8
L.S.D. (P = 0.05)		16.8	22.1	13.5

discernable in Petri-plate studies, this was particularly so with linoleic and oleic acid additions; L. saeva produced close to tissue development with the two latter supplements. The purple pigment produced by these species tended to become concentrated into the dispersed droplets of these fatty acids.

Propionic, valeric, oleic and palmitic acid produced a non-significant elevation in yield of the L. nuda isolate. Ethanol valeric acid and acetaldehyde produced a non-significant growth increase in L. saeva, this species in Petri-plate studies showed best development with ethanol, oleic and palmitic acid additions at c. 1g/L .

High yields by C. gambosa (Table 9.1) on butyric, acetic and valeric acids were through development of sectors. Growth in Petri-plates with palmitic acid, linoleic acid and acetaldehyde supplements at c. 1g/L also caused the development of sectors.

10:0 GROWTH RESPONSE TO A RANGE OF LEAF AND MANURE EXTRACTS AS SOURCES OF GROWTH FACTORS (FIGURE 10.1)

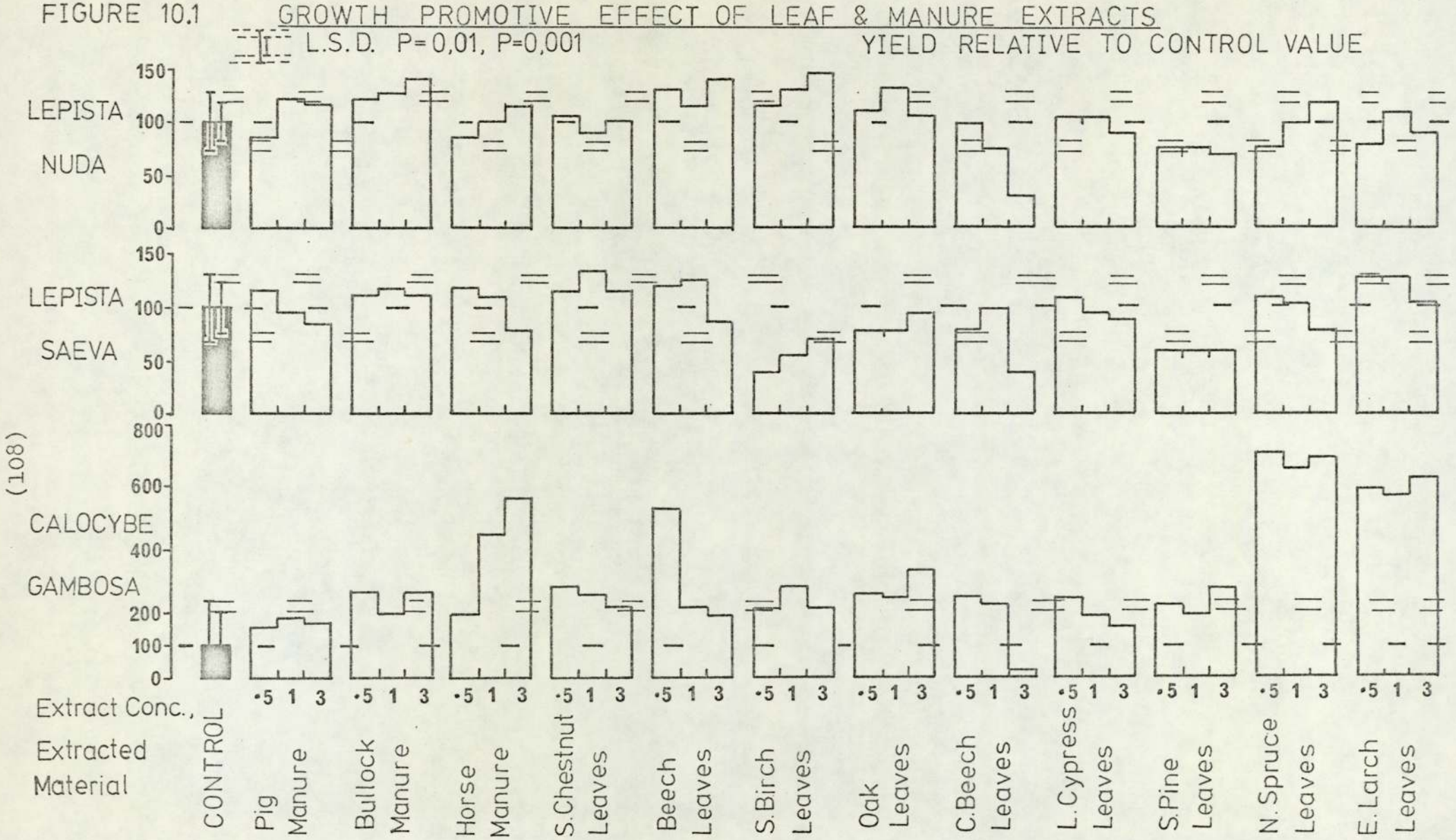
Growth by L. nuda isolate 7 was very significantly ($P = 0.001$) improved with additions of extracts from beech (0.5 &3.0 mg/L) birch (1.0 &3.0 mg/L) and oak (1.0 mg/L) together with bullock manure extract (3.0 mg/L) compared to the unsupplemented control value.

Significant ($P = 0.01$) elevation in yield occurred from addition of pig (1.0 g/L) and bullock manure (0.5 &1.0 mg/L) extracts.

L. saeva isolate 6B demonstrated a very significant growth increase only on media supplemented with sweet chestnut leaf extract, significantly improved values were obtained with beech leaf (1.0 mg/L) and larch leaf (0.5 &1.0 mg/L) extract additions .

Highly significant increases in dry-weight in relation to the control value were achieved by the C. gambosa isolate following addition of a spectrum of extracts. Larch and spruce leaf extracts were particularly potent in their ability to stimulate growth at all levels

FIGURE 10.1 GROWTH PROMOTIVE EFFECT OF LEAF & MANURE EXTRACTS



of addition. Horse manure stimulated yields in rough proportion to its level of addition, best growth with beech leaves was produced at the lowest level of addition. The leaf extracts produced from deciduous trees were broadly active, extracts from Scotch pine and Lawson's cypress were considerably less active than those from Norway spruce and European larch. Pig manure did not cause a significant growth promotion.

It is probable that growth factor(s) for C. gambosa were contained in a number of these extracts.

Copper beech at 3.0 mg/L addition was inhibitory to growth of all species.

SECTION 5

11.0 ASSESSMENT OF CELLULOLYTIC ABILITY WITHIN AND BETWEEN SPECIES

Clearing of an opaque ball-milled cellulose medium was assessed for two concentrations of cellulose in agar columns and by comparison, in relation to colony growth on agar discs in Petri-plates (Table 11.1). The former technique indicates the net cellulolytic activity of colonies where there is a physical limitation on extension growth, the latter method, which is considerably less precise, demonstrates zones of clearing beneath actively growing colonies.

The L. nuda isolates demonstrated a considerably weaker cellulolytic ability than the range of L. saeva isolates, mean values were approximately one-half those obtained by the latter. A large component of the variability in the standard error of the mean for the L. saeva strains was due to the poor cellulolytic ability of isolates C.B.S. 355.33 and 6A, the latter isolate was obtained in deciduous leaf-litter in a similar situation to that in which L. nuda frequently grows.

Clearing by L. nuda strains was generally in localised regions within the colony on Petri-plates giving rise to pear-shaped or semicircular regions where clearance was complete, but frequently mottled regions of incomplete clearing. L. saeva strain 6A also demonstrated

TABLE 11.1

SPECIES ISOLATE NO.	TUBES				PETRI - PLATE	
	0.25 per cent		1 per cent		1 per cent	
	AGAR VOLUME CLEARED (cm^3)	CELLULOSE UTILISED AS A % of* 0.0375g	AGAR VOLUME CLEARED (cm^3)	CELLULOSE UTILISED AS A % of 0.0375g*	AGAR VOLUME CLEARED (cm^3)	CELLULOSE UTILISED AS A % of 0.2g**
LEPISTA NUDA						
1	4.2	28	1.2	32	3.2	16
247.69	4.7	31	1.7	45	4.4	22
5	2.5	17	2.1	56	3.8	19
6			0.86	23	0.53	3
7	7.3	49	1.1	30	4.9	25
8	3.4	23	0.86	23	8.4	42
9	4.7	31	.86	23	1.1	6
10			1.2	32	4.9	25
11	3.0	20	1.5	40	3.8	19
		28.4 ± 4.0		33.8 ± 3.8		19.7 ± 3.8
LEPISTA SAEVA						
1	7.3	49	3.3	88	15.6	78
355.33	4.2	28	1.3	35	11.7	59
248.69					17.7	89
5	8.4	56	3.3	88	11.7	59
6A	4.7	31	1.3	35	†	
6B	12.0	80	3.7	99	11.0	55
7	15.0	100	3.0	80		
8	14.0	94	3.4	91		
		62.6 ± 11.0		73.7 ± 10.2		68.0 ± 6.6
CALOCYBE GAMBOSA						
1	5.2	35	Tr.		Tr.	
2	5.4	36	Tr.		Tr.	
		35.5				

* Weight of Cellulose in 15 ML 0.25% Cellulose Agar

** Weight of Cellulose in 20 ML 1.0% Cellulose Agar

† Clearing of Cellulose in v. Localised Regions.

Tr Trace Clearing (Indistinct Zone).

the formation of clearance zones only in restricted areas of the colony growing in Petri-plates. These characteristics are indicitive of the development of secunds.

Clearing by L. saeva strains was generally very distinct in Petri-plates, extending in a complete circular zone from the colony centre to a zone of partial clearing at the boundary of the colony, suggestive of a uniform production of cellulase throughout the colony.

Clearing of cellulose agar by the two C. gambosa isolates was apparent only at a concentration of 0.25 per cent, although clearing and some growth did occur in Petri-plates at 1 per cent, this did not extend through the depth of the agar and therefore its limits could not be clearly defined.

12:0 ASSESSMENT OF P-DIPHENOL OXIDASE LEVELS WITHIN AND BETWEEN SPECIES

The strength of the Bavendamm 'oxidase' reaction was variable within the L. nuda and L. saeva species, the C. gambosa isolates tested produced a clear indication of p-diphenol oxidase activity on both gallic and tannic acid substrates (Table 12.1).

The L. nuda strains 6, 9, 11 & 13 obtained from isolates growing in soil showed strong oxidase activity, it is possible that those strains inhabiting the lower layers of leaf-litter (at the litter-soil boundary zone) would possess higher levels of phenoloxidase activity than strains colonising recently fallen litter.

The oxidase reactions of L. saeva isolates all (except 6A) formally growing in pasture land soils did not, however, exhibit high levels.

Growth of all L. nuda and L. saeva isolates tended to be strongly or totally inhibited on the gallic acid medium, improved growth and more pronounced discolouration was shown by a number of the isolates of both species with the tannic acid medium.

Formation of a cherry-red zone beneath the colonies and inocula of the C. gambosa isolates and L. saeva strain 1 may indicate

TABLE 12.1

SPECIES	ISOLATE NO.	GALLIC ACID OXIDASE RTN	GALLIC ACID GROWTH	TANNIC ACID OXIDASE RTN	TANNIC ACID GROWTH
LEPISTA NUDA	1†	+	-	+	PI
	247.69	+	-	+	PI
	493.48	+	-		
	300.58	+	-		
	5	+	-	++	PI
	6	++	SI	++	SI
	7	(+)	-	+	SI
	8	(+)	-	+	PI
	9	++	SI	++	PI
	10	+	-	+	NI
	11	++	SI		
	12			+	NI
	13			++	PI
LEPISTA SAEVA	1†	+	-	++	SI
	248.69	+	-	+	SI
	355.33	++	NI	++	NI
	5*	++	NI	++	NI
	6A	+	PI		
	6B*	+	-	+	-
	7*			+	Tr
CALOCYBE GAMBOSA	1†	++	NI	++	NI
	2†	++	NI	++	NI

KEY

	OXIDASE REACTION		GROWTH
(+)	Discolouration Confined to Inoculum	-	No Growth
+	Discolouration in Inoculum and weakly in zone beneath	Tr	Trace growth on Inoculum
++	Strong discolouration of medium	SI	Strongly Inhibited
*	Inoculum v. strongly discoloured	PI	Partly Inhibited
†	Discolouration as a Distinct Brown-Cherry-Red zone on Tannic Acid Medium	NI	No Inhibition.

the presence of intermediate breakdown products of tannic acid; clearing of the agar beneath the fungal mat of L. saeva strain 355.33 as distinct from a discoloured peripheral zone may indicate utilisation of tannic acid or its breakdown products as carbon sources.

SECTION 6

13:0 GROWTH IN SOLID SUBSTRATES

SPECIES	ISOLATE NO.	GROWTH-RATE (mm/day)	OBSERVATIONS ON GROWTH AND MORPHOLOGY IN TUBES AND FLASKS
LEPISTA NUDA	1	1.47	Fine mycelial development. Poor growth. Dark colouration of compost following growth suggests poor utilisation. No stranding. Development in tubes of a few dense fluffy white patches in compost and at compost-casing interface.
	247.69	3.42	Mycelial density medium. Strong strand development in casing, tendency to formation, in flasks, of densely packed fine white particles at compost casing interface.
	5	3.84	Dense uniform mycelial growth. Some strand development in casing. Good utilisation of compost.
	6	1.93	Slow growth. Development of some strands in casing and compost.
	7	3.8	Dense uniform growth. No strand formation.
	8	3.62	Medium density mycelial growth. Marked tendency towards strand development in compost.
	9	3.76	Strong, though fine, mycelial growth in compost and casing, strong lilaceous colouration particularly at the casing surface in flasks. Good utilisation of compost (assessed by colour following growth). Stranding in flasks, marked tendency to strong development of strands in the compost of all tubes.
	10	3.93	Dense, fine, white hyphal development through compost. Dark colour of colonised compost following incubation, indicitive of poor utilisation. Slight strand development in tubes. Formation of compact balls of hyphae in the compost of the flasks.

SPECIES	ISOLATE NO.	GROWTH RATE (mm/day)	OBSERVATIONS ON GROWTH AND MORPHOLOGY IN TUBES AND FLASKS
LEPISTA NUDA	11	1.92	Strong growth in compost and casing, this is carried almost to the extent of tissueing in the flasks, with a bright purple colouration of hyphae especially at the casing surface. Development of strands in the casing of both tubes and flasks. Strong development of strands in the compost of one tube. Dark brown/black stain from tissued hyphae pressed against the flask-wall at one point, is a possible indication of strong tyrosinase activity.
LEPISTA SAEVA	1	2.52	Dense, though slow, mycelial growth. Good colonisation, though dark colour of compost following several months incubation suggests poor utilisation. Some strand development in compost of tubes. In flasks numerous small, compact balls of hyphae developed (These spheres had brown centres indicating tyrosinase activity).
	248.69	2.36	Dense slow growth, through the compost, resulting in colonisation almost to the extent of tissueing in flasks. Cultures in tubes showed strong development of strands in the casing, but to a greater extent in the compost. Growth through the casing was generally weak.
	5	3.16	Dense white mycelial growth. Compact tissueing along straws and occasionally as balls of hyphae in flasks, with formation of dense white particles scattered in the upper region of the compost in tubes.
	6A	1.57	Medium dense though very slow mycelial growth in tubes, somewhat stronger development in flasks with unusually strong growth through the casing layer. No strand development.

SPECIES	ISOLATE NO.	GROWTH RATE (mm/day)	OBSERVATIONS ON GROWTH AND MORPHOLOGY IN TUBES AND FLASKS
LEPISTA SAEVA	6B	2.97	Medium dense mycelial growth. Some thick strand formation in tubes. Light brown appearance of compost indicates good utilisation. All flasks and tubes contain numberous white compact balls of hyphae, 1 mm dia., in tubes, larger in flasks c.2 x those formed by isolate 1 in flasks. Small dense regions also apparent in tubes (possibly sectors)
CALOCYBE GAMBOSA	1 2		Tubes inoculated with both strains 1 & 2 did not support growth.

Observations were made on the growth-rate of single L. nuda and L. saeva isolates (No. 1) on a range of formulated 'short-composted' substrates (Table 13.1), relative to rate of growth achieved on a commercial mushroom growers compost. The substrates were subdivided, one-half underwent a secondary thermophilic fermentation (peak-heat) and pasturisation. Analysis for total Kjeldahl nitrogen and moisture was made.

TABLE 13.1 Growth on Short Composted Substrates.

SUBSTRATE †		ANALYSIS		Lepista nuda 1	Lepista saeva 1
Non-Peak Heated (N-PH) Peak Heated (PH) (Pasteurised)		g % N (Kjeldahl Nitrogen)	Moisture %	per cent of control	per cent of control
Commercial mushroom compost (control)	PH	2.3	65	100	100
Short composted Barley Straw and Chicken manure	N-PH	2.44	79	52	67
	PH	2.32	77	Tr	71*
Short composted Barley straw, chicken manure and wood shavings	N-PH	1.92	76	89*	119*
	PH	2.02	75	82	79*
Spent compost, wood shavings, and compost activator.	N-PH	1.76	67	82*	83
	PH	1.72	66	54	48
Spent compost, wood shavings, malt dust compost activator.	N-PH	1.53	63		
	PH	1.67	71	63	75
Wood shavings, chicken manure, compost activator	N-PH	1.68	70		
	PH	1.57	78	75	52
Wood shavings, spent compost, chicken manure and compost activator.	N-PH	2.13	66	75*	63*
	PH	1.74	65	54	52
Wood shavings grass cuttings.	N-PH	0.7	76	68	71*
	PH	1.73	71	75	74
Wood shavings and compost activator	PH	0.99	68	82*	103*
Spent compost (obtained from beds of the cultivated mushroom)		1.94		Ingredients forming the larger proportion of each substrate are listed first.	
Malt Dust		0.462	Dry		

TABLE 13.1 cont:

SUBSTRATES Non-Peak Heated Peak Heated (Pasteurised)	ANALYSIS g % N (Kjeldahl Nitrogen)	ANALYSIS Moisture %	
Wood shavings. (Pine or spruce shavings formally used as stable litter and partly rotted)	1.1	73.1	Ingredients forming the larger proportion of each substrate are listed first.
Chicken manure (Broiler chicken litter containing wood shavings)	3.35	66.2	

† Duplicate tubes for each substrate were autoclaved (see materials and methods) and inoculated individually with a few grains of the appropiate grain spawn.

* Dense hyphal growth of isolates. The Lepista saeva strain on these substrates produced strong strand development leading to compact balls of hyphae in the surface compost adjacent to the inoculum (possible primordia).

SECTION 7

14:0 FRUITBODY DEVELOPMENT ON A NATURAL AGAR MEDIUM

C. gambosa isolates 2, 3 and 5 and *L. saeva* isolates C.B.S. 248.69, 5 and 6B produced only vegetative growth over the agar surface. *C. gambosa* isolates 3 and 5 during incubation produced large numbers of orange stellate crystals on the colony surface and finally autolysis took place. *L. saeva* strain 6B produced well defined radiating strands from the central inoculum to peripheral sites of growth, but did not show further development.

L. nuda isolate 13 strongly colonised the agar following twenty-one days incubation forming a dense development of purple aerial hyphae, but showed no differentation. *L. nuda* isolate 8 produced strong hyphal growth over the agar medium. At five months following inoculation the colony surface developed dense aggregations of hyphae, possible fruitbody initials, however, these did not show structural differentation nor did they develop further.

L. nuda isolate 9 formed primordia and small immature fruitbodies in the third month following inoculation initially at the mycelial-agar interface along the sides of the flask (Plates 11–19). The sporophores, the largest of which attained a size of 10-15 mm, produced a reddish-brown pileus and speckled lilaceous stipe, supporting a number of thick, soft, well spaced lamellae. Microscopic examination revealed no discernable hymenium and basidia - no spore print was produced. Development was gymnocarpic, the immature fruitbodies differentiated from the terminal portion of stalk-like growths, with the larger specimens the base of the stipe expanding downwards to encompass the supporting structure.

Large numbers of branched stalk structures which had the appearance of antler-like growths developed in the area around the sides of the flask where the agar had shrunk back from the flask walls. Small immature sporophores were generally produced from the terminal portions of these branches. Dense masses of unbranched stalk structures were produced in areas of the main colony on the upper agar surface during the fourth month, however, these structures did not differentiate further.

LEPISTA NUDA FRUIT-BODY PRIMORDIA FORMED ON MALT/YEAST EXTRACT AGAR.

PLATE 11.

Development of twin primordia between the agar and flask-wall.

SCALE 1 cm.

PLATE 12.

Primordial development on the agar 'wall' within the gap created by contraction of the agar.

SCALE 1 cm.

PLATE 13.

Fruit-body primordia 'pinched off' from the distal part of stalk-like growths formed from the surface hyphae.

SCALE 1 cm.


50

LEPISTA NUDA FRUIT-BODY PRIMORDIA FORMED ON MALT/YEAST EXTRACT AGAR.

PLATE 14.

Indication of the large number of primordia which formed in each flask.

SCALE 1 cm.

PLATE 15.

Well developed primordia from several flasks.

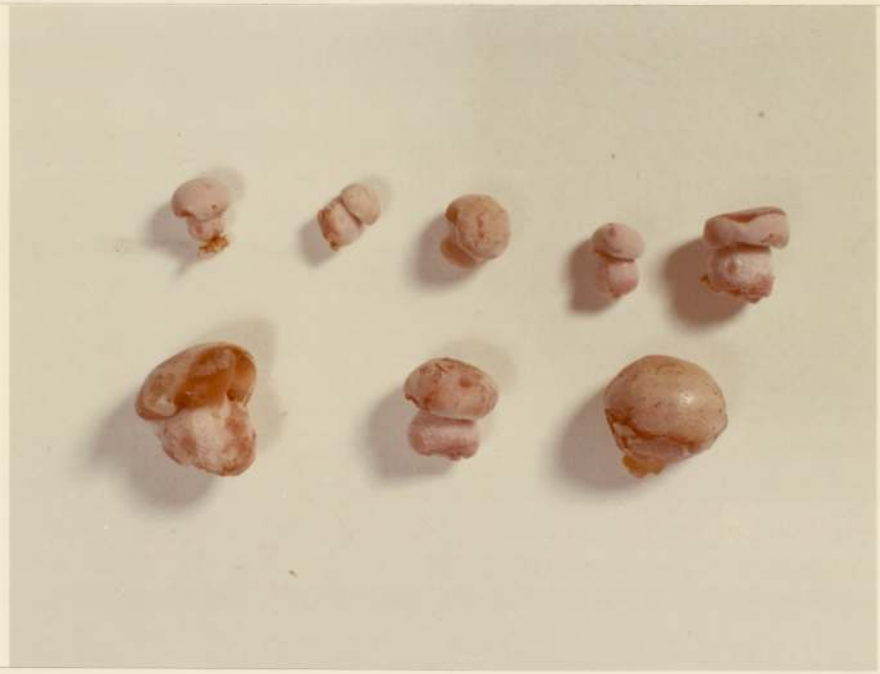
SCALE 1 cm.

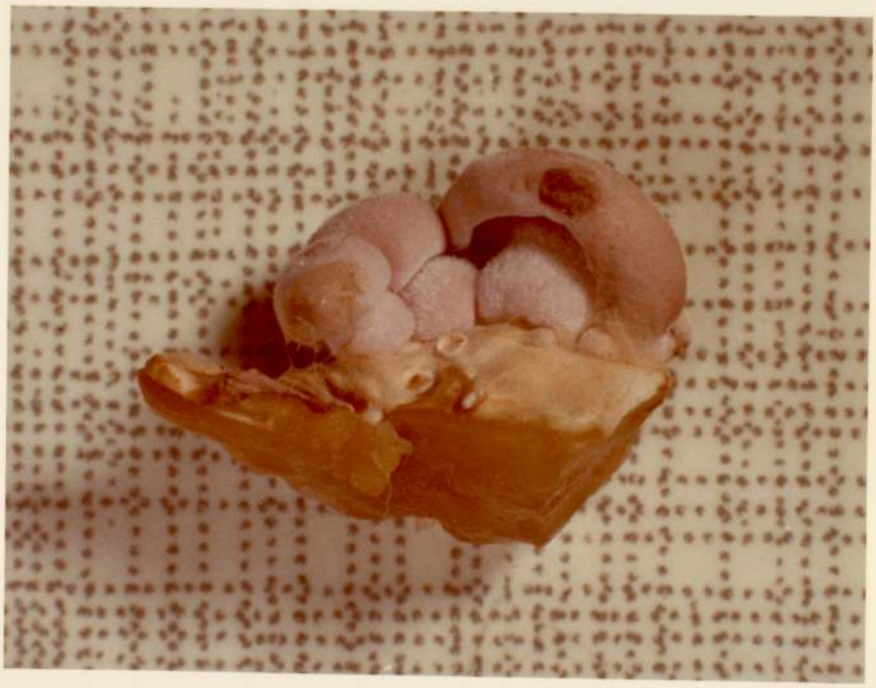
PLATE 16.

Clump of 3 primordia. Lamellae at early stage of development.

SCALE 1 cm.


150
APPROX.
VOLUMES
100
50





LEPISTA NUDA FRUIT-BODY PRIMORDIA FORMED ON MALT/YEAST EXTRACT AGAR.

PLATE 17.

Primordia developing from the terminal region of branched 'antler-like' growths. (Arrowed).

SCALE 1 cm.

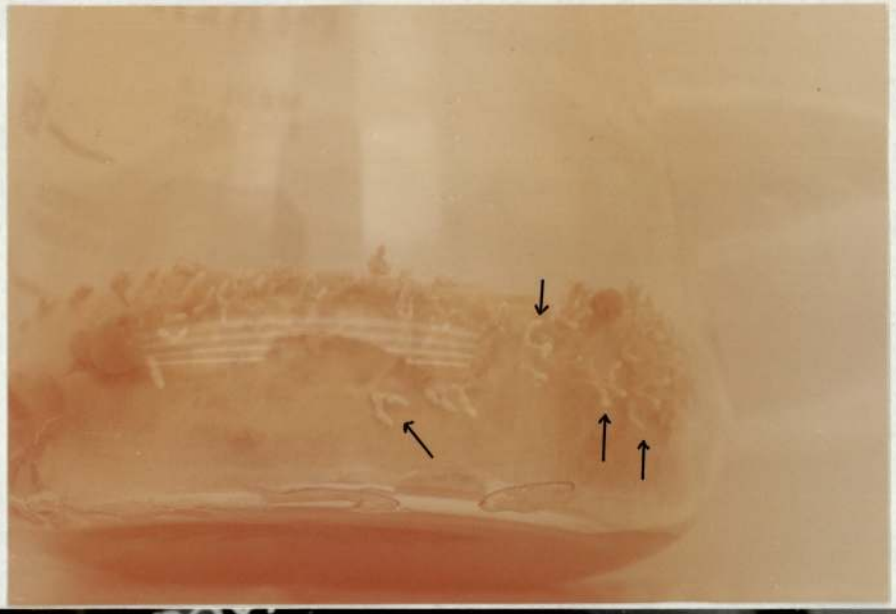
PLATE 18.

and

PLATE 19.

Differentiation of numerous primordia.

SCALE 1 cm.




APPROX.
VOLUMES
100
50

In several of the flasks submerged masses of externally purple pigmented tissue, c.1 cm diameter, developed within the volume of agar, causing splitting of the agar, however, the formation of these structures in the 5th month occurred too late for any direct correlation with the main phase of sporophore development.

14.1 Fruitbody Formation on a Solid Substrate

All three L. saeva isolates colonised the compost and casing layers, particularly strong growth through the compost was made by 1 and 6B, these isolates also made best progress with growth through the casing materials together with some development of strands within both compost and casing. Growth into the casing layers was considerably less vigorous than that achieved by L. nuda, in this respect the peat-chalk casing mix and loam soil proved to be the most suitable materials.

Colonisation by the L. nuda isolates within the compost progessively intensified, mycelial density was not initially as marked as that achieved by L. saeva strains. Growth from the colonised compost into the leaf preparations, peat-chalk and soil casing layers was generally vigorous and persistent. Over a period of several weeks following colonisation of the two successive layers of casing materials, in each case the L. nuda isolates formed a dense surface layer of purple tissued hyphae. This hyphal development was possibly analogous to the 'dense, flakey and crusty' growth referred to by Constantin and Matruchot .

L. nuda isolates 8 and 9 produced fruitbodies in each of the four treatment containers (Plates 20-28), isolate 6 produced only a single fruitbody.

Dissection of a container of isolate 8 shortly following the advent of fruitbody production revealed 15 fruitbodies at various stages of maturity. The fruitbodies generally arose within the 1st casing layer, frequently at the compost interface, and also at the sides of the container where contraction in volume of casing and

P L A T E 20.

Lepista nuda sporophore produced in culture. (Isolate No.8).

Note: Strong development of surface purple hyphae.

SCALE 3 cm.

P L A T E 21.

Sporophores of Lepista nuda, (Isolate No.9), formed in culture. Abnormally developing fruit-bodies produced from a central mass of tissue.

SCALE 1 cm.

P L A T E 22.

Sporophores of Lepista nuda. (Isolate No.9 left; Isolate No.8 right from Plate 9 at an earlier stage).

SCALE 4 cm.

PLATES 23 & 24.

Lepista nuda Isolate 8.

23. (Left) Sporophore emerging from side of container.

SCALE 1 cm.

24. (Right). Sporophore (from 12 above) removed from container, showing large mass of basal tissue.

SCALE 1 cm.

PLATES 25 & 26.

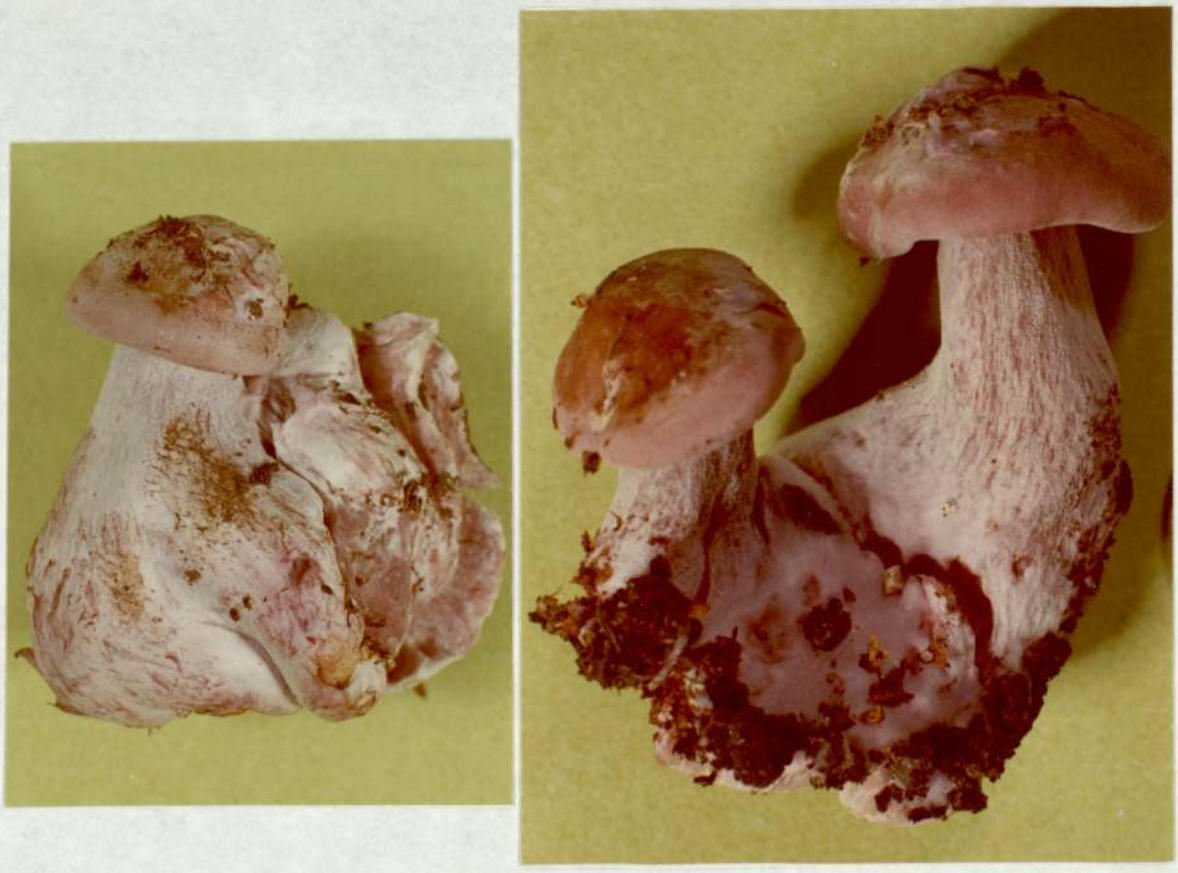
Lepista nuda Isolate 8 .

25. (Left). Immature sporophore associated with large mass of basal tissue.

SCALE 2 cm.

26. (Right) Immature sporophores with strongly developed colouration.

SCALE 2 cm.



P L A T E 27.

3 Fruit-bodies of Lepista nuda (Isolate 9) with relatively long stipes and small caps. Note: 'Foot' comprising basal tissue mass.

SCALE 2 cm.

P L A T E 28.

Malformed sporophore of Lepista nuda (Isolate 9), associated with a mass of immature sporophores. These further developed to give those of Plate 10.

SCALE 1 cm.

compost had caused a gap to appear. Fruitbodies arising centrally required to breakthrough the surface 'skin' of hyphal tissue; this surface layer greatly restricted water uptake by the casing, applied over the casing surface, and limited free gaseous diffusion, its presence was probably of importance in producing conditions within the casing conducive to fruitbody formation.

All immature fruitbodies were associated with a bulb of tissue (Plates 25 & 26), in one case five immature fruitbodies arose from a single common bulb of tissue, generally the stipe increased in width towards the base forming a continuation with this basal bulb. The tissue mass became integrated into the base of the stipe as the fruitbody enlarged and matured, giving rise to the 'blue-foot' which appeared as an extension of the stipe generally at an obtuse angle to the main body of the stipe (Plate 24).

Strong development of strands occurred within the casing layer during fruitbody formation. Those strands associated with the base of the developing sporophores were particularly large and probably constituted the rhizomorphs referred to by Constantin and Matruchot.

The small fruitbodies left to mature enlarged very considerably in size from 1-1½ inches to a height of c.5 inches, sizes equivalent to those found in nature. Allowed to expand the fruitbodies produced the typical recurved cap and 'tricholomatoid' appearance attaining eventually unnaturally large proportions, a phenonmenon recorded as gigantism by Constantin and Matruchot.

A number of fruitbodies in one container of isolate 9 were formed atypically (Plate 21). These arose from a central mass of tissue. A number of the more mature fruitbodies developed a stipe curvature sufficient to invert the cap. In those specimens where the cap made contact with the basal tissue, fusion occurred at the point of contact, where contact was made with the hyphae on the casing surface, strong development of several thick strands occurred at that point.

Fruitbodies allowed to develop produced a mature hymenium from which a number of spore prints were made.

Grain Spawns

Generally good colonisation of grain spawn media was achieved by L. nuda and L. saeva isolates.

The suitability of the grain spawn media was roughly related to its physical properties. Rye proved superior in this respect due to the hardness of the grain preventing splitting during cooking procedures, and size, in terms of convenience in washing and dispersal as an inoculum. Wheat grain was prone to splitting, exudation of starch causing clumping of grain, sticking to the base of the flask and considerably increased chances of contamination. Millet was less manageable in washing and soaking procedures, the inoculum consisted of small compact aggregations of millet grain and mycelium. The milo grain included a large proportion of split grains which were difficult to eliminate and causedfree starch formation, milo grain proved unsatisfactory for this reason.

SECTION 8

Effect of Oxygen and Carbon dioxide tension on hyphal growth: Experiments with the gas diffusion column

15:0 INFLUENCE OF OXYGEN TENSION

The direct effect of oxygen on the rate of mycelial growth of two isolates each of L. nuda (7 & C.B.S. 247.69) and L. saeva (5 & C.B.S. 248.69) was studied for gas mixtures grading in composition from atmospheric (21.0% oxygen) to 0.3% oxygen (Figures 15.1 & 15.2). The lower level represented the maximum level of this gas as an impurity in the supplies of commercial nitrogen. Nitrogen gas was run against air, producing a countercurrent flow, in which the nitrogen acted as an inert dilutent replacing the oxygen as its concentration fell along the length of the column.

In general maximal growth was attained at very low concentrations of oxygen. The oxygen concentration at which growth

FIGURE 15.1
EFFECT OF OXYGEN CONCENTRATION ON GROWTH RATE

0-21 PER CENT OXYGEN

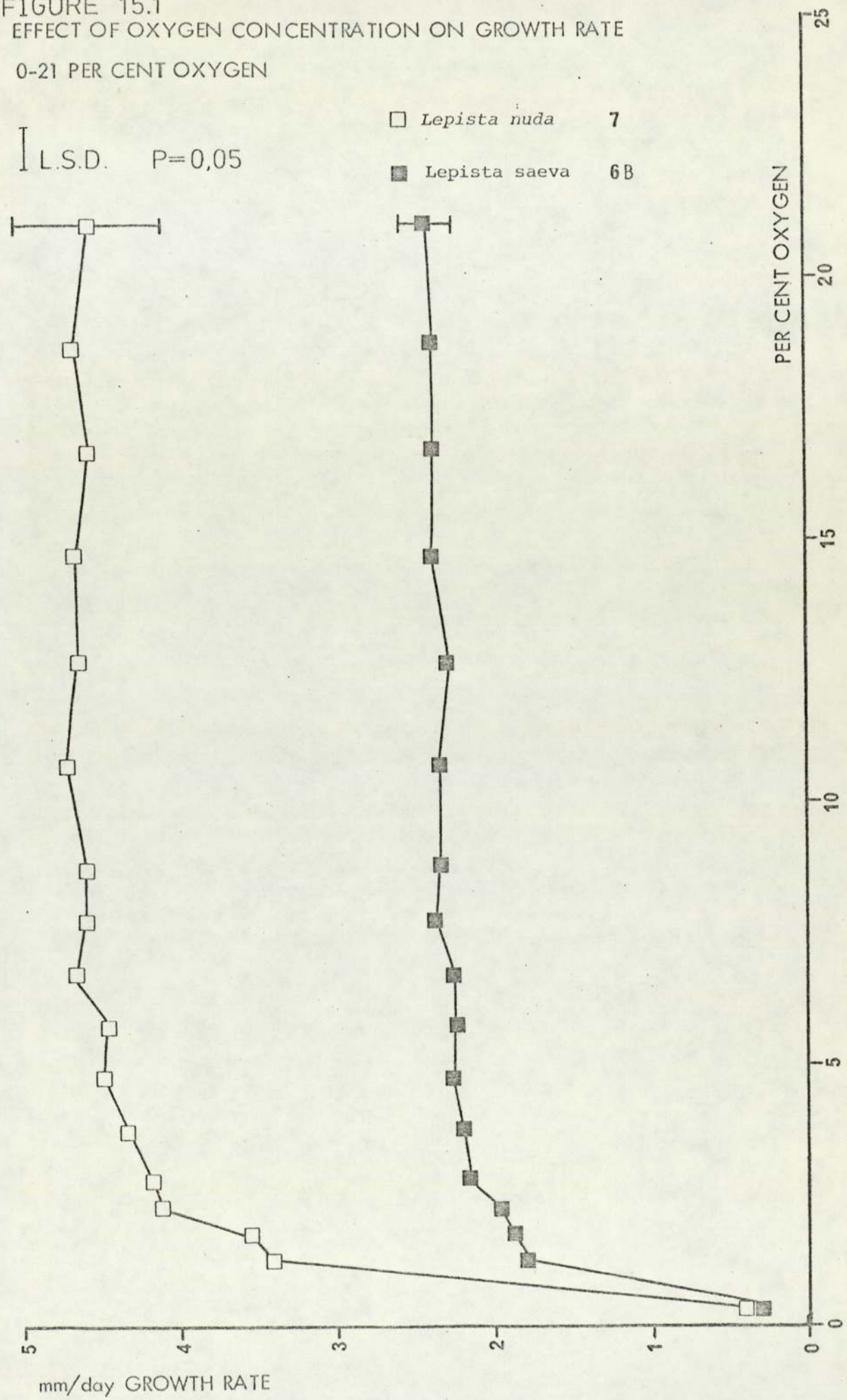
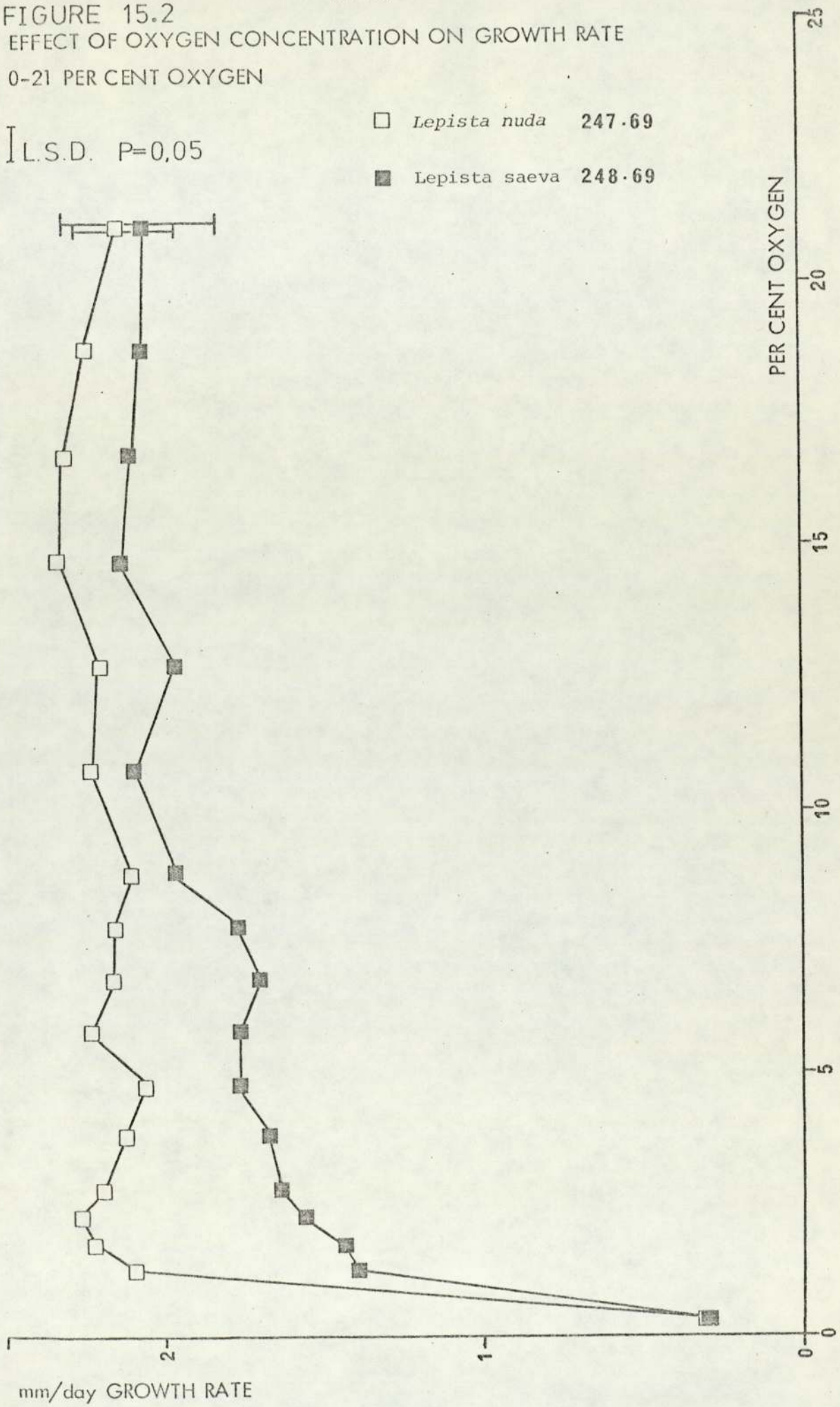


FIGURE 15.2
EFFECT OF OXYGEN CONCENTRATION ON GROWTH RATE
0-21 PER CENT OXYGEN



was reduced to half maximal is considered to be important in determining those oxygen concentrations which would act as an effective constraint to growth in nature. Low oxygen tension could occur in water films, in water-logged soils and at the core of soil crumbs or larger soil aggregates.

Maximal growth of L. nuda isolate 7 was maintained until an oxygen concentration of 2.8%, at this value growth was significantly ($P = 0.05$) reduced to that achieved at atmospheric levels (21.0%). A reduction to half maximum growth rate was produced at c. 0.8% oxygen. Only slight aerial hyphal growth was produced at 0.3% oxygen.

Growth rate of the isolate C.B.S. 247.69 was approximately half that of isolate 7 on the glucose-asparagine agar medium. Near maximal growth was achieved with a reduced oxygen tension of 1.25%, growth at this value was just significantly ($P = 0.05$) below that at atmospheric levels. Half maximal growth was produced at similar values, c. 0.7% to that causing 50% growth inhibition in isolate 7.

L. saeva strain 5 demonstrated a significantly inhibited growth rate ($P = 0.05$) at 4% oxygen. A reduction to half the rate of colony extension occurred at c. 0.9% oxygen. No growth took place at 0.3% oxygen. Isolate C.B.S. 248.69 showed a significant ($P = 0.05$) reduction in rate of hyphal extension at approx., 8.0% oxygen in comparison with that at atmospheric levels. A gradual and progressive reduction in growth-rate from maximal values may indicate instability of this isolate under oxygen stress, however, the oxygen concentration required to retard growth to half maximum was very low at approximately 1%, a similar value to that obtained for isolate 5. Some aerial hyphal development off the inoculum plug occurred at 0.3% oxygen.

In conclusion growth of both species was not significantly inhibited until oxygen concentration was reduced to 4%. That concentration causing a 50% reduction in growth may be considered to be a more reliable measure of the tolerance of the fungus to variation in oxygen concentration. This value for all isolates was in the region 0.7 - 1.0% oxygen. Slight growth was apparent for some isolates at 0.3% oxygen.

15:1 Influence of Carbon Dioxide Tension

Growth response to higher than atmospheric levels of carbon dioxide (0.03%) was considerably more variable than that produced by reduced oxygen tension.

L. nuda C.B.S. 247.69 and L. saeva C.B.S. 248.69 were cultured in atmospheres containing carbon dioxide in the range 0.69 to 12.0% (Figure 15:3). Carbon dioxide replaced nitrogen in the gas mixture. Significant (P = 0.05) inhibition of the L. nuda isolate was evident in the approximate concentration zone 3.2 to 6.12% CO_2. Growth inhibition between 6.0 to 10.5% CO_2 showed a linear reduction directly proportional to increase in carbon dioxide concentration. Fifty per cent inhibition of growth was produced at a carbon dioxide tension of 9.5%.

A significant (P = 0.05) reduction in growth of the L. saeva isolate occurred between 4.5 to 5.0% CO_2 with marked growth inhibition beyond 9%. Half maximal rate of growth was produced with a carbon dioxide level of 10.6%.

Rates of hyphal extension for L. nuda isolate 7 and L. saeva isolate 6B were determined over a range of carbon dioxide tensions between zero and five per cent (Figure 15.4).

Growth inhibition of L. nuda 7 was marked above *c.* 2.0%, maximum growth was recorded at 0.35% CO_2, however, this was not significant with respect to zero per cent. Considerable growth inhibition was apparent over the range 0.35 to 3.0% CO_2, at values above 3% growth rate was not strongly affected. At 0.6% CO_2 growth was significantly (P = 0.05) inhibited in relation to the zero per cent value. Fifty per cent inhibition of growth was produced at 2.4% CO_2.

Growth reduction of the L. saeva isolate 6B was more directly proportional to carbon dioxide concentration. Growth was significantly (P = 0.05) reduced from maximal at 0.7%, however, a 50% reduction in growth rate was only achieved at values in slight excess of 5% CO_2.

FIGURE 15.3

EFFECT OF CARBON DIOXIDE CONCENTRATION ON GROWTH RATE
0-12 PER CENT CARBON DIOXIDE

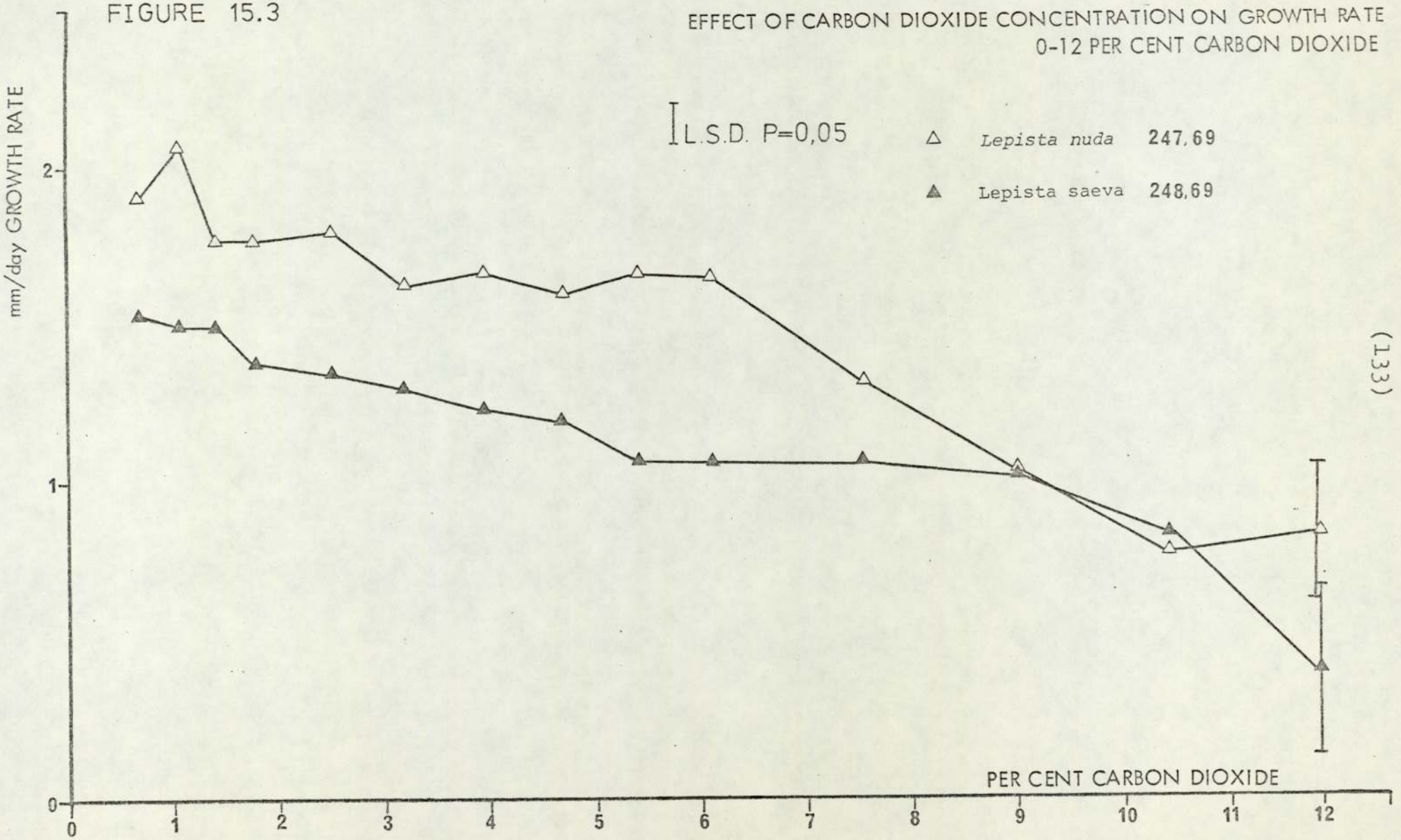


FIGURE 15.4

EFFECT OF CARBON DIOXIDE CONCENTRATION ON GROWTH RATE
0-5 PER CENT CARBON DIOXIDE

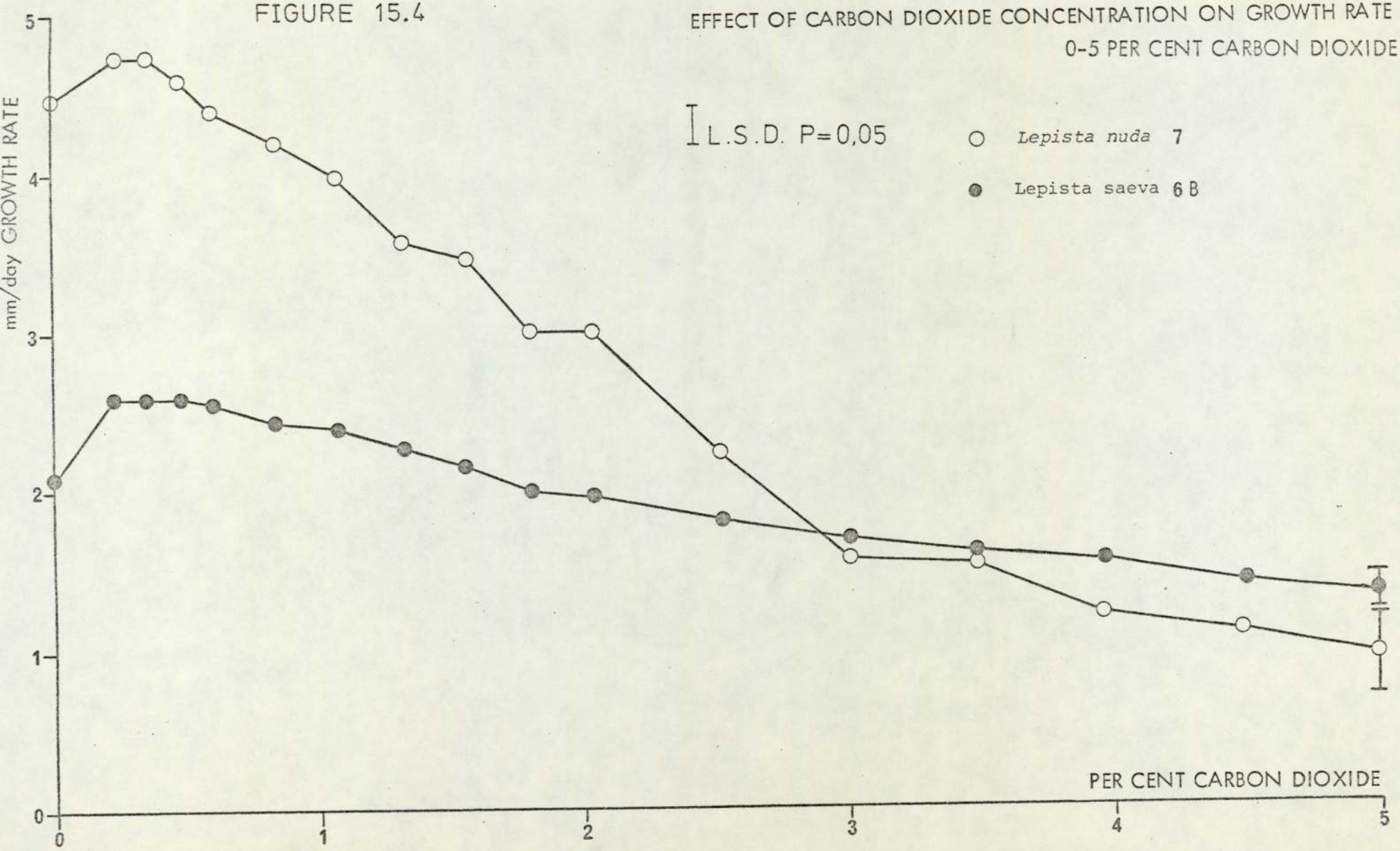
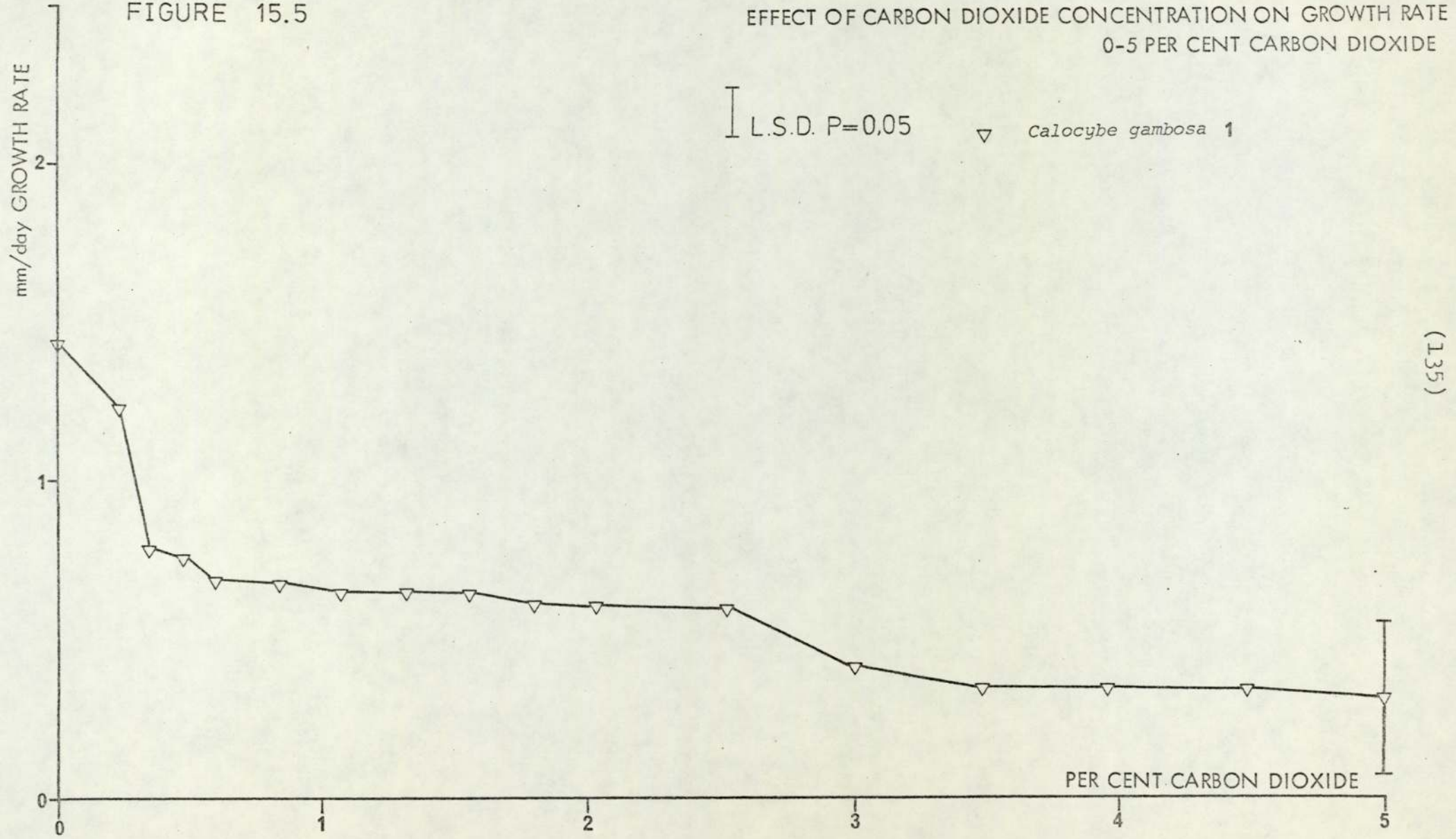


FIGURE 15.5

EFFECT OF CARBON DIOXIDE CONCENTRATION ON GROWTH RATE
0-5 PER CENT CARBON DIOXIDE



Growth response for a single isolate of Calocybe gambosa (I) was ascertained at carbon dioxide concentrations with the range 0-5% (Figure 15.5). Growth of this strain was strongly inhibited at 0.6% CO_2, growth rate was significantly inhibited (P=0.05) at 0.25% CO_2. A reduction to half maximum growth rate occurred at 0.55% CO_2.

In conclusion the inhibiting effect of carbon dioxide was progressive during incubation, growth inhibition rated over long incubation periods was cumulative as represented by the growth rates given here; inhibition at low carbon dioxide tensions was only clearly apparent following 25 days incubation of the L. nuda and L. saeva isolates at 0-5% CO_2.

In assessing the effect of carbon dioxide on growth of cultures, factors which also require consideration in addition to colony age, include nutrient status of the cultures, hyphal density and growth rate. The faster growing and more dispersed colonies of L. nuda 7 were more susceptible to inhibition by carbon dioxide than the slower developing and denser cultures of L. nuda C.B.S. 247.69 and L. saeva C.B.S. 248.69.

DISCUSSION

AND

CONCLUSIONS

DISCUSSION

The present study was undertaken to assess the physiological requirements of the three species L. nuda, L. saeva and C. gambosa for vegetative growth as a prerequisite to an assessment of the additional nutritional and environmental requirements forthe formation of fruitbodies.

The high mycelial dry-weights obtained for L. nuda and L. saeva are in sharp contrast to the very low yields given for growth of these species on synthetic media by a number of previous investigators (Kőgl and Fries 1937, Modess 1941, Norkrans 1950, 1953, Lundeberg 1970). Explanations for yields below 0-15mg frequently obtained are difficult to provide. It is considered that although the isolates obtained by these authors were generally from coniferous locations and may have been less adaptable to growth in synthetic media, the isolates showed a general inability to utilise the media components provided. Separate sterilisation and addition of carbon and nitrogen components, preferably with sterilisation achieved by filtration, was stressed during this study; autoclaving sugars and phosphates or sugars and nitrogen sources is known to promote formation of inhibitors (Cochrane 1958), and associated detrimental effects including hydrolysis of carbon compounds or deamination of nitrogen sources and exhaustion of buffer capacity.

Initial experiments showed that L. nuda and L. saeva were strongly inhibited where glucose with asparagine or glucose with ammonium tartrate were autoclaved together in media (yields below 35 mg following 30 days incubation). The studies of Lilly and Barnett (1951) have indicated that autoclaving glucose-asparagine medium at neutral or alkaline pH has resulted in the precipitation of important metallic ions including Fe, Al, Ca, Ba, Mg, Mn and Zn from the medium. Although it is not suggested that in all these studies combination of such factors is the sole explanation, it is of interest to note that Lamb (1974) obtained considerably improved yields to these investigators with an L. nuda isolate from a pine plantation in a simple synthetic medium where these precautions in sterilising media components were strictly adhered too.

In general the denser hyphal growth shown by L. saeva isolates was reflected in higher dry-weights than those achieved with L. nuda strains on the most favourable sources of carbon and nitrogen. Highest yields of L. saeva isolate 6B were up to one-third in excess of those obtained for L. nuda isolate 7 on the best carbon and nitrogen sources. This is clearly shown from the yields obtained in the C:N ratio studies on the standard glucose-asparagine medium (Figures 6.3, 6.4, 6.5 & 6.6). The yields obtained in C:N ratio studies and analysis for residual sugar following growth of L. saeva 6B on a range of amino-nitrogen sources (Table 8.1) indicate that this species was able to produce good growth until the majority of carbon and nitrogen in the medium was utilised, in this respect efficiency of growth exceeded that of the corresponding L. nuda strains.

The Economic Coefficient is determined from the formula:

$$\frac{\text{Mycelial dry weight (g)}}{\text{Carbohydrate consumed (g)}}$$

Values derived for L. nuda isolate 7 from carbon: nitrogen ratio studies indicate an E.C. of 0.4 - 0.5, these figures approximately agree with those obtained for Lambs (1974) isolate of 0.45 - 0.55 on the best carbon sources. Both sets of figures assume complete utilisation of the carbon source. The E.C. value for the L. saeva isolate (6B) derived from yields on amino-acid nitrogen sources where analysis for residual glucose was made is 0.73, this value agrees well with a figure of 0.71 obtained from C:N ratio studies, where complete utilisation of sugar is assumed. High Economic Coefficients are generally a feature of Basidiomycetes (Burnett 1968).

Carbon Sources

Comparison of growth between L. nuda and L. saeva isolates over a wide range of carbon and nitrogen sources suggested a higher level of nutritional adaptability by L. nuda. L. nuda isolate 7 produced better growth than the corresponding L. saeva isolate 6B on the carbon sources pectin, galactose and the polyols dulcitol, mannitol and sorbitol (Figures 7.1 & 7.2). Highest dry-weights were produced on the hexitols derived from the sugars supporting

heaviest yields (i.e. mannose-mannitol, glucose-sorbitol), L. saeva produced markedly improved growth on ribose in comparison with L. nuda. Both species appear to possess a wide-range of enzymes enabling utilisation of disaccharides including sucrase (invertase) maltase, cellobiase, trehalase and melibiase. Raffinose is split either to fructose and melibiose by sucrase or to galactose and sucrose by attack at the alternative linkage. The high yields obtained on raffinose in comparison with poor utilisation of galactose and melibiose by these species, when present as sole carbon sources, indicates that provision of an alternative carbon source by attack on raffinose provides energy for induced enzyme systems required for metabolism of galactose and melibiose.

It is probable that these species can synthesise the sugars mannitol, trehalose and glycogen which have been detected in significant quantities in the mycelium and fruitbodies of Agaricus bisporus (Hughes 1961, Holtz 1971, Hammond and Nichols 1976).

Both species produced good growth on starch indicating strong production of α & β amylases. Dense colony mats were produced in the combined starch + maltose + glucose medium, combination of pectin, galactose and glucose improved growth for L. nuda but not for L. saeva. Combination of ribose, arabinose and xylose did not improve their utilisation by either species, growth on mannose, sorbose and arabinose approximately equalled the expected yield on the mannose fraction.

Poor utilisation of the organic acids, citrate, malate tartrate, succinate and oxalate is general within the fungi, the exception being good growth obtained by Agaricus bisporus on these Krebs cycle acids (Bohus 1959). Large amounts of oxalic acid may be accumulated by this fungus (Tsao 1963). Inability to utilise these substances is probably due to their inaccessibility caused by formation of insoluble Na or Ca salts, or impermeability due to ionisation of the cell-wall, cell membrane or cell colloids in the normal physiological pH range. Improved utilisation may occur at low pH values (Cochrane 1958).

Competitive inhibition between the sugars glucose and fructose and mannose, glucose and xylose has been recorded for T. nudum by Reusser, Gorin and Spencer (1960) and Cirillo and Grayson (Cirillo 1961). The sugars are preferentially utilised in the order stated.

The C. gambosa isolate generally produced best yields on the hexose sugars and disaccharides present (Figure 7.3). Xylose proved a favourable carbon source and good growth was produced on the combination ribose, arabinose and xylose. Yields given represent actual mycelial dry-weights produced and do not include weight of the inoculum mycelial-agar-plug which has been frequently included in yield tables in the literature, this may give a mistaken impression of the extent of growth when the actual yields recorded are low.

Cellulolytic Activity

All species showed moderate yields where cellulose was present as sole carbon source. Cellulolytic ability was assessed for a number of strains within each species as the capacity of the fungus to lyse finely divided cellulose producing zones of clearing in a milk-white opaque medium. The results (Table 11.1) indicated considerable variation within the respective groups (L. nuda range 17 to 49%; L. saeva range 28 to 100%; as unit values on 0.25% cellulose agar). Clearing could only be assessed for the C. gambosa isolates on 0.25% cellulose addition.

This method provides a convenient means of assessing cellulolytic capacity for a large number of fungi with adequate replication of individual treatments. Ability to breakdown 72 hr ball-milled cellulose which is present in a finely divided state cannot be directly correlated with attack on native cellulose. Organic nitrogen sources such as asparagine and yeast extract included in the medium may reduce the extent of cellulase production (Malik 1970). The extent of clearing will also be directly related to the density of the mycelial mat developing above the agar, in this

respect results may be bias in favour of greater cellulase production by L. saeva. Growth and cellulose clearing in Petri-plates, however, indicates that L. saeva produces overall higher levels of cellulase. Initial experiments in which L. nuda and L. saeva isolate 1 were grown on beech and spruce sawdust agars with synthetic salts addition indicated rapid surface hyphal growth and slow clearing of the agar.

The inability of certain investigators (Reusser, Spencer and Sallans 1958 b, Lundeberg 1970) to demonstrate cellulolytic ability by L. nuda isolates may be due to only slight mycelial development by their strains under the conditions of culture. Norkrans (1950) and Lindeberg (1946) have been able to demonstrate cellulose breakdown by all three species following incubation on sterilised Glyceria straw.

Polyphenol Oxidase Enzyme Levels

Assessment of polyphenol oxidase activity as a measure of an individual species capacity to degrade lignin also indicated variability between strains (Table 12.1), this is most apparent in considering the inhibitory effect on growth of gallic and tannic acid or their breakdown products. Discolouration of gallic and tannic acid media in the Bavendamm test is considered to be due to the production of ortho-and para-quinones or quinone-protein complexes (Lindeberg and Fåhraeous 1952, Lindeberg and Holm 1952). The formation of intermediate orange coloured complexes observed for C. gambosa and single isolates of L. nuda and L. saeva has been observed for Agaricus bisporus (Stoller, 1954) a strong producer of polyphenol oxidases.

Production of a coloured zone on gallic and tannic acid media is considered to indicate p-diphenol oxidase or laccase activity (Walker 1975, Lindeberg 1948). The properties of tyrosinase and laccase enzyme fractions which constitute the polyphenol oxidase component of Basidiomycetes can be summarised as:

(a) Tyrosinase, oxidises monophenols (i.e. Tyrosine, p-cresol) and ortho-diphenols (i.e. catechol, pyrogallol). Oxidation of tyrosine leads to the formation of black melanin pigments.

(b) Laccase, oxidises ortho-diphenols and para-diphenols (i.e. para-phenylamine diamine, guiacol). This enzyme is sensitive to inactivation by carbon monoxide.

The level of individual enzyme systems is generally assessed by colour reactions on mono-phenols (tyrosine, p-cresol) indicating only tyrosinase activity and on para-diphenols (p-phenylaminediamine, guiacol) indicating only laccase activity and occassionally by the use of carbon monoxide selective for the presence of laccase. Discolouration on gallic and tannic acid and on catechol does not clearly differentiate between the contribution made by each enzyme system, however, tyrosinase is considered to be intracellular and laccase extracellular, the Bavendamm test has been demonstrated to show the presence of extracellular laccase (Lindeberg 1948).

It has been demonstrated for Agaricus bisporus that most of the tyrosinase is present in the fruitbodies, the laccase is in the mycelium within the compost, both enzymes have been detected within the strands of the casing layer. (Lindeberg 1950, Turner 1968, 1974). The presence of a tyrosinase system in L. nuda (isolate 7) and L. saeva (isolate 6B) was indicated by the development of black pigments where tyrosine was employed as sole nitrogen source. Black discolouration of submerged mycelium of C. gambosa was also observed. All three produced a strong black discolouration when catechol was added to the culture filtrates. It is suggested that C. gambosa possesses a very strong laccase system but weak tyrosinase production. These enzyme levels appear to be more equally balanced in L. nuda and L. saeva.

Matsubara and Iwaski (1972) have indicated fairly high levels of tyrosinase and laccase in fruitbodies of Lepista subnuda. The laccase enzyme is probably adaptive in nature (similar to cellulase), studies with A. bisporus on a commercial mushroom growers compost have indicated gradually increased levels of laccase until the point at which fruitbody production and maturation occurs (Turner 1967, 1975).

The studies of Waksman and Nissen (1932), Gerrits, Bels-Koning and Muller (1962) and Gerrits (1967) have shown that the

cultivated mushroom, Agaricus bisporus, obtains the majority of its nitrogen when grown in commercial 'synthetic' and horse-manure composts from degrading the fixed (insoluble) nitrogen-rich-lignin-humus complex (that lignin fraction insoluble in 72% sulphuric acid), which has been built-up by fixation of ammonium nitrogen into amino-acids and peptides by the action of micro-organisms during the composting cycle. Ability to degrade the 'lignin-humin' complex within the upper layers of forest and pastureland soils may provide an important source of organic nitrogen compounds for fungi colonising these layers. These substances would be unavailable to fungi lacking polyphenol oxidase enzyme systems.

'Start Glucose'

The importance of 'start glucose' has been indicated by the researches of Lilly and Barnett (1951), Norkrans (1950) and Lamb (1974). 'Start glucose' consists of a trace glucose addition to the medium which is considered to supply sufficient energy for the activation of 'adaptive' enzyme systems as opposed to the normally operative 'constitutive' enzyme systems. These enzymes may include enzymes which split oligo-or polysaccharides at the cell surface or carry these substances(permeases) across the cell membrane for intracellular lysis. Several such enzyme systems are known to have a requirement for glucose (Burnett 1968).

Lamb (1974) obtained highly significant ($P = 0.001$) increases in yield following addition of trace glucose on the carbon compounds galactose, fructose, maltose, dextrin, glycogen, starch and inulin for his strain of L. nuda, the isolate was apparently unable to accumulate sufficient energy for growth over the 42 day incubation period on these substrates alone for induction of adaptive enzymes to occur. The inclusion of asparagine in the basal medium used in the present studies may have enabled adaptation to a number of carbon sources, however, very little growth was made in control flasks where asparagine constituted the sole carbon and nitrogen source, it is unlikely therefore at the concentration at which it was present it

constituted a significant energy source.

It is probable that repeated subculture of the present isolates on 2% malt extract agar which contains significant levels of maltose, glucose, sucrose and dextrin ensured that the enzyme systems required for the utilisation of these substrates were active, however, Lamb's cultures were isolated onto 2% malt extract and routinely cultured on Hagem's agar, which contains 5g/L malt extract, his isolate of L. nuda was able to show only minimal growth on maltose, sucrose and dextrin. It is possible that the minimal medium employed by Lamb which was deficient in a range of inorganic ions (Na, Zn, Ca, Mn and Cu) could have restricted utilisation of some of these carbon compounds.

Nitrogen Sources

The results for the growth of the three species on organic, ammonium, nitrate and nitrite nitrogen-sources indicate that final yields must be considered in relation to pH change in the medium (Figures 8.1-8.3). Utilisation of a few organic nitrogen sources resulted in a net release of ammonia into the medium, which was clearly demonstrated with the metabolism of urea. Poorer growth of L. saeva isolates on urea compared with L. nuda was probably a result of less tolerance of this species to alkaline conditions. Release of ammonia may also occur particularly at high nitrogen levels with metabolism of amide and amino-acid nitrogen sources. This is demonstrated by a shift to higher pH values by L. nuda 8 and L. saeva 5 on propionamide and with C. gambosa isolates on asparagine. A shift to alkaline pH occurred with all species at high asparagine concentrations in C:N ratio studies (Figures 6.1 & 6.2). Metabolism of metallic nitrate salts generally resulted in a shift to alkaline conditions (L. saeva 5 & 6B on pot. nitrate and 5 on sodium nitrate), however, this may be countered by organic acid formation in the medium resulting in a stable pH. This may have part attributed to high yield by L. nuda isolates 7 & 8 and L. saeva 5 on nitrates.

Utilisation of ammonium compounds is recognised to result in the formation of acid conditions. This is particularly so

with metabolism of ammonium salts of strong acids, good growth on ammonium bicarbonate by all species probably resulted from the formation of a weak acid (carbonic acid) with consequent slight effect on pH. The use of combined ammonium salts (i.e. ferric ammonium citrate, magnesium ammonium phosphate or ammonium hydrophosphate) is advantageous in preventing a pH fall with utilisation of the ammonium ion (Bohus 1959) or combination of organic acids as carbon source with ammonium salts to counter the downward pH drift (Cochrane 1958, Nicholas 1965).

All three species utilised nitrate, good utilisation showed a tendency to be strain specific, ammonium was metabolised in preference to nitrate (Nicholas 1965). Growth was inhibited by nitrite, Rawald (1963) recorded his isolate of L.nuda as showing some growth on sodium nitrite, however, the growth produced relative to the nitrogen-free control value was sufficiently slight for this finding to be ignored.

Calocybe gambosa isolates produced best development on the compound sources of amino acids, peptone and casein hydrolysate. High yields were produced by isolate 1 on asparagine, this isolate also generally grew well on ammonium and nitrate nitrogen. Ammonium tartrate proved a moderate source of nitrogen to both, however, poor yields resulted on ammonium bicabonate and urea. This may have been due to toxicity caused by the release of free ammonia.

Comparison of yields of single isolates of L.nuda and L.saeva on a range of 19 amino-acids, amide and compound sources of amino-acids (Figures 8.4 & 8.5) indicated a wider nutritional diversity by the former species, similar to the pattern shown on carbon sources. Nine amino-acids enabled the achievement of high yields by L.nuda, five by L.saeva, those proving unsatisfactory for the latter species were valine, leucine, threonine and arginine. The amides asparagine and glutamine were equally good sources of nitrogen for L.nuda, glutamine compared very poorly with excellent yields achieved by L.saeva on asparagine. The compound sources of amino-acid nitrogen, casein hydrolysate and the amino-acid mix were comparable with asparagine for L.nuda, however, the amino-acid mixture was distinctly less favourable for L.saeva in comparison with casein hydrolysate.

The majority of amino-acids are considered to be converted directly into proteins (Cochrane 1958), however, where amino-acids or amides form the sole source of nitrogen those best able to take part in transamination reactions would be expected to produce highest yields. Asparagine best fulfills the transamination requirement. Casein hydrolysate and the amino-acid mix are most readily converted into protein.

Poor utilisation by L. saeva (isolate 6B) on glutamine and the amino-acid combination was probably due to a rapid fall in pH with their utilisation, possibly due to the formation of oxaloglutaric acid or similar Krebs cycle acids. Metabolism of aspartic and glutamic acids by both species resulted in a shift to higher pH values. Development of acid conditions following metabolism of arginine probably also accounted for low yields by the L. saeva isolate. Metabolism of arginine within the ornithine cycle possibly resulted in the formation of fumarate or associated organic acids resulting in the pronounced pH disturbance.

The C. gambosa isolate produced best development on the compound sources of nitrogen, the amino-acid mix and casein hydrolysate (Figure 8.6). Highest yields amongst the amino-acids were formed on valine and phenylalanine. Glutamic acid and glutamine produced slightly improved growth to that attained on the corresponding amino-acid and amide, aspartic acid and asparagine. Glutamate has been attributed as a growth factor for Tricholoma gambosum (Norkrans 1953)

C:N Ratio

The carbon:nitrogen ratio of natural substrates is difficult to assess due to their inherent heterogeneity; an optimum C:N ratio in vitro was determined for L. nuda and L. saeva of c. 21:1 (Figures 6.2-6.6), a higher ratio is indicated for C. gambosa (Figure 6.1). L. saeva demonstrated an ability to grow with low levels of nitrogen in the medium.

The optimum C:N ratio for vegetative growth may be defined as, "that balance of carbon and nitrogen, at a specific concen-

tration, and in relation to other components of the medium, which places no restriction on metabolism and cell synthesis required for maximal rates of hyphal/ growth". Growth is considered as increase in cell size, weight and number, and is for the purposes of this definition measured as increase in dry-weight. This requirement is a direct reflection of the organisms genetic resources and is related to the organisms ability to obtain and maintain certain minimal proportions of cell components (intracellular 'pools'). Extrapolation to the organisms natural enviroment is difficult, where there are many potential sources/ of cell components with varing levels of accessibility and priority in pathways of utilisation within the cell.

In pure culture an optimum C:N ratio will be related directly to the conditions of culture (solid, semi-solid, stationary liquid or submerged),/ physical conditions prevailing within these culture systems (aeration, agitation,/ temperature) and the duration of the incubation period, for each particular organism. Two important considerations arising from the present studies are the requirement for an individual C:N ratio for each combination of carbon and nitrogen sources where these form the sole source of these elements, and the choice of a C:N balance providing a stable optimum physiological pH throughout/ out the duration of the incubation period, a requirement which is related to approximately equal depletion of carbon and nitrogen sources in the medium. The ability of Tricholoma nudum to synthesise protein or fats in submerged culture has been clearly demonstrated to be related to the level of carbon and nitrogen in the medium (Reusser Spencer and Sallans 1958b).

Influence of Temperature

Isolates of L.nuda and L.saeva examined for effect of temperature/ on growth-rate indicated a generally lower temperature optimum (20°) than previously reported. Temperature profiles and optima appear to be more variable for strains of L.nuda, the good growth apparent for both species at low temperatures (10°C) with some growth at 4°C is consistant with activity/ of these species at low temperatures during winter months; fruiting may be stimulated by light frosts, fruitbodies of L.saeva are known to have been formed while snow is on the ground. The strong staling of five L.saeva isolates/

at temperatures of 27-28°C indicates that this species is unlikely to withstand high surface soil temperatures. The single isolate of C. gambosa tested demonstrated a temperature optimum at 25°C, growth was markedly reduced at 10°C and absent at 4°C.

Influence of Hydrogen Ion Concentration

Growth profiles over a range of hydrogen ion concentrations indicated an optimum for L. nuda at neutral pH and for L. saeva at slightly acid values (c. pH 6.0), the former species showed more tolerance of increased acid or alkaline conditions. C. gambosa indicated a preference for slightly acid conditions.

Vitamin Requirement

Assessment of the growth response of these species to a series of deletions from a complete vitamin solution indicated a clear requirement for thiamine. This is general within the saprophytic hymenomycetes (Lindeberg 1946). Pyrimidine has been demonstrated to be the essential component for Tricholoma nudum and T. gambosum (Norkrans 1950). In addition a partial requirement for biotin and possibly also pyridoxine and folic acid is indicated for L. nuda and L. saeva. Growth of C. gambosa isolates in this series appeared to be suppressed possibly by choline chloride and inositol additions to the complete vitamin mix, or the use of a charcoal purified asparagine preparation.

Metal Ion Requirements

The complete synthetic medium proved generally superior to the basal solution and equivalent to the basal solution plus leaf litter or manure ash additions. Supplementation of the basal solution with the synthetic mineral solution or zinc generally suppressed growth, calcium at trace addition slightly removed the inhibition caused by zinc. It is probable that the calcium component of the ash additions and the high level of calcium in the complete medium enabled high yields by antagonism of the effects of high levels of metal ions (suppression of the inhibition produced by zinc is indicated from the data) or metabolic staling substances

accumulating in the media. An absolute requirement for calcium is therefore not indicated by growth on the basal solution from which it was absent, however, addition of calcium is advantageous for reduced growth inhibition of these species under the conditions of high nutrient status and direct proximity to accumulating toxic metabolities associated with stationary liquid culture.

Ecological Considerations

L. nuda isolate 8 was less demanding in its nutritional requirement than the corresponding isolate 7, in nitrogen source, vitamin and trace-metal requirement studies. This strain showed only slightly inhibited growth on the tannic acid medium

The three species L. nuda, L. saeva and C. gambosa may represent three stages in the degradation of dead plant tissues. L. nuda can be considered to occupy a position as a primary saprophytic coloniser within the basidomycete flora. The fungus is able to rapidly colonise partly rotted natural or artificial accumulations of deciduous (commonly beech leaves) or coniferous leaves, and is able to make sufficient growth coupled with tolerance of low temperatures to accumulate sufficient reserves for fruitbody formation prior to the onset of heavy frosts and freezing temperatures. Lignin decomposing strains able to degrade the more resistant plant material in the soil beneath the surface leaf-litter accumulations are suggested to have arisen which colonise organically rich soils. The tolerance of some strains to phenolic materials in plant leaves appear to enable them to colonise the less readily attacked residues (i.e. accumulations of coniferous leaves). Lepista nuda emerges as a genetically adept and nutritionally resourceful fungus able to make use of readily degraded materials (proteins, pectin, starch hemicelluloses) and able to slowly utilise the more resistant fractions (cellulose and lignin).

Lepista saeva typically occurs in pastureland soils and is probably closely associated with the decomposition of roots, stems and leaves within the grass sward. It occasionally inhabits positions

in open copses. L. saeva strains were generally slower growing than those of L. nuda on the composted substrate, however, hyphal growth was more dense. This species demonstrated a stronger cellulolytic ability than L. nuda.

Calocybe gambosa similarly to L. saeva is able to form a perennial mycelium which typically gives rise to fairy-rings. It occupies a similar habitat to the latter also occasionally arising within open woodland. Nutritional data indicates that this species is able to strongly decompose lignin, but its cellulolytic capacity is comparatively weak. The ability to breakdown the resistant 'lignin-humin' fraction of soils may enable this fungus to gain access to a variety of nitrogeneous materials, possibly including amino acids, peptides and proteins complexed to the lignin polymer. C. gambosa produced slow growth in pure culture, best growth was obtained in natural media (malt extract, potato dextrose agar) which may be due to a requirement for certain growth factors (high levels of such growth factor(s) are indicated to be present in the leaves of spruce and larch) and for organic nitrogen compounds. These media also have a high carbon:nitrogen ratio.

Mycorrhizal v. Saprophytic status

The nutritional status of mycorrhizal fungi is currently under review (Ferry and Das 1968, Palmer and Hacskaylo 1970, Lewis 1973, Lamb 1974) and also the relative contribution of the commonly associated hymenomycetes to the mycorrhizal flora, has been questioned (Zak and Marx 1964, Lamb and Richards 1970), however, although the nutritional limitations of the mycorrhiza former appear not as restricted as formally considered (Melin 1925) - the possibility of a passive saprophytic existence has been envisaged in the absence of the host plant - the nutritional diversity shown by these three species together with the ecological data are almost certainly sufficient to place them in a solely saprophytic category.

Influence of Oxygen and Carbon Dioxide Tension on Growth

Data from the effect of oxygen concentration on hyphal growth of L. nuda and L. saeva isolates indicated no growth inhibition

until oxygen concentration is reduced to 2-4%. Growth rate is only markedly inhibited at 0.7 to 1.0% at which concentration growth is reduced to approximately half maximal. Oxygen deficiency is therefore unlikely to be a serious hazard for growth of these species in soils and leaf-litter. Oxygen would be limiting only in very heavy compacted or water-saturated soils.

Carbon dioxide at low concentrations (5%) exerted a direct toxic effect on isolates of L. nuda, L. saeva and C. gambosa. Inhibition of growth was most pronounced for two fast growing L. nuda and L. saeva isolates, half maximum growth resulting at values of 2.4% and 5% CO_2 (the growth curve of this L. saeva isolate (6B) was similar to that produced for a second isolate (5) in a repeat experiment). Two slower growing isolates on the synthetic glucose-asparagine medium at 16°C, produced denser colony mats and were less susceptible to inhibition by carbon dioxide. Half maximal growth resulted at 9.5 and 10.6% CO_2 for L. nuda and L. saeva strains respectively. It is suggested that isolates colonising surface leaf-litter (a 'sub-aerial' enviroment), or producing surface growth in light soils and generally producing a more rapidly developing and dispersed mycelium, are relatively more susceptible to the antibiotic action of carbon dioxide than those strains inhabiting deeper soil layers, producing a denser more perennial mycelium. The former instance would be considered to contain L. nuda, the latter L. saeva. For both species, however, the susceptibility to carbon dioxide is considered to be a variable factor within species, relating directly to the ecology of the original isolate.

The growth of the cultivated mushroom, Agaricus bisporus is retarded by carbon dioxide concentrations below 10% (Tschierpe 1960, Nair 1972) although there is some discrepency as to the extent of this inhibition which may be related to strain characteristics or conditions of culture (i.e. humidity, temperature, media composition). Agaricus bisporus is able to thoroughly colonise highly compacted horse manure-straw 'composts' under commercial conditions, without a discernable reduction in growth-rate, this contrasts with poor development of L. nuda and L. saeva strains, following relatively light compaction of

the commercial mushroom growers compost used in solid substrate fruiting trials. Accumulation of respired carbon dioxide to inhibitory levels appears to be the factor responsible.

Carbon dioxide at low concentrations (< 1.0%) markedly inhibited the growth of C. gambosa strain 2. C. gambosa has a high incidence of occurrence on basic soils particularly calcareous clay supporting rough grassland (often grassy banks and roadside verges), it has also been recordedon leaf-litter of birch and ash in open woodland (University of Birmingham, Herbarium records).

Within the aqueous phase of alkaline soils carbon dioxide is largly converted via carbonic acid to bicarbonate which further dissociates at high pH to form carbonate ions (Ponnamperuma 1967, Griffin 1972). The relationship is simply expressed by the equation:

$$H_2O + \underset{\text{gaseous}}{CO_2} \rightleftharpoons H_2CO_3 \overset{+H_2O}{\rightleftharpoons} H_3O^+ + HCO_3^-$$

$$HCO_3^- \overset{+H_2O}{\rightleftharpoons} H_3O^+ + CO_3^{2-}$$

Carbon dioxide may also combine with calcium and magnesium carbonates in aqueous solution (Ponnamperuma 1967).

Dissociation of carbonic acid to form bicarbonate and carbonate ions is directly influenced by hydrogen ion concentration. The importance of the carbon dioxide, carbonic acid, bicarbonate equilibrium to fungal physiology was recognised in early studies (Garrett 1938); more/
recent experimental evidence indicates that bicarbonate is metabolically active at alkaline pH (Griffin 1965, MacCauley and Griffin 1969). The conversion of carbon dioxide to bicarbonate in basic soils results in lower partial pressures of carbon dioxide in these soils relative to acid soils assuming equivalent respiratory rates (Griffin 1972). It may, therefore, be assumed that fungi inhabiting alkaline soils are less exposed to direct toxicity by accumulation of gaseous carbon dioxide through the existence effectively of a bicarbonate sink.

With reservations that the data concerning the effect of carbon dioxide on C. gambosa is limited to a single isolate cultured on an artificial nutrient rich medium (2% malt extract agar), and therefore extrapolations to growth in nature require to be tentative, it is probable that for certain strains growth in nature may be restricted to surface leaf-litter and soil layers, light soils and to alkaline soils due to direct toxicity by carbon dioxide at low concentrations. (0.5 - 1.0%).

Fruitbody Development

A measure of success was achieved in obtaining immature fruitbodies for an isolate of L. nuda in pure culture on a semi-solid medium and fruitbodies of three strains on a non-sterile composted substrate.

Development of fruitbodies in pure culture occurred following 9 weeks maintained at 12°c with a circadian light cycle of 9 hrs light, 15 hrs dark, supplied by fluorescent daylight tubes. Hyphal development over the surface of the agar was promoted by an initial 3 week dark incubation period at 25°c. The culture system consisted of 100 ml quantites of 2% malt extract (Boots Pure Drug Co.,) + 0.1% Yeast extract (Oxoid Ltd.,) solidified by 1.5% agar addition (Oxoid No. 3) forming a c. 2cm deep layer at the base of 250 ml wide mouth Erlenmeyer flasks, plugged with bleached non-absorbent cotton-wool.

Mycelial aggregations representing possible primordia were detected for four strains of L. saeva within the peat chalk capping employed in the growth tubes to prevent desiccation of the agar inoculum.

The L. nuda isolate (9) forming fruitbodies on an agar medium was probably more genetically predisposed towards their development under this system of culture. This phenomenon is characteristic of some strains of Agaricus bisporus which readily produce primordia in pure culture (Hume and Hayes 1972, Angeli-Couvy 1975).

Fruitbody formation probably arises as a direct consequence of a check in hyphal growth (Cochrane 1958). Limitation of growth is

considered to favour formation of reproductive structures (Hawker 1950, Plunket 1953). Work conducted by Hawker with Melanospora destruens indicated 0.5% glucose was optimal, the general finding that oligo-and polysaccharides are more suitable than simple hexoses as sources of carbon for formation of reproductive structures was related to ability of the organism to obtain a constant low level of hexose sugar, this was generally the case for carbon sources which were poorly utilised by the fungus (i.e. cellulose). Ease of phosphorylation was considered to be a critical factor in determining their effect upon fruiting (sucrose was favoured in this respect), the level of phosphorylated hexoses was considered to directly affect respiration rate and hence available energy for chemical synthesis.

High levels of nitrogen generally inhibit fruiting. Formation of reproductive structures does not appear to be favoured by any particular source of nitrogen. The mineral requirement though probably increased is not generally considered to be extended compared to the requirement for vegetative growth, with the possible exception of copper as a component of tyrosinase required for spore pigmentation or calcium acting in an antagonistic capacity. Vitamin nutrition for fruitbody development may be more heterotrophic, there is evidence (Cochrane 1968) of an increased requirement for thiamine and biotin. The suitability of 2% malt extract + 0.1% yeast extract agar upon which fruitbodies were obtained may reflect its ability to fulfil most of these nutritional requirements.

Large numbers of small fruitbodies of L. nuda appear to be able to form where there is only a small amount of substrate provided, this was also observed following colonisation of spent tan bark in plant pots by Constantin and Matruchot (1898). It is not clear whether there is a minimal nutritional requirement for their development, however, further development of more advanced fruitbodies (i.e. those better disposed to obtain in a flow of nutrients from the mycelium) appears to be only possible where the substrate bulk is increased as was the case with the composted substrate in containers or in leaf-piles by Constantin and Matruchot. This mechanism is similar to that first elucidated for Coprinus lagopus (C. cinereus) by Madelin (1956), with L. nuda however, the increased bulk of the sporophore may

delimit a minimum nutritional requirement for primordium formation. This requirement for fruiting on agar may account for a considerably lengthened incubation period prior to fruitbody formation (3 months) or to a minimum substrate level met by the employment of an increaseddepth of agar to that employed for Petri-plate studies. The use of an increased bulk of agar may have a buffer effect against the processes of desiccation and staling experienced with semi-solid media during long term incubation studies. A similar volume of malt extract based agar (Hagem agar modified after Modess, 1941) and flask culture system, has been successfully employed in cultural studies concerning fructification of a number of species of Boletaceae where incubation has continued over a period of several months (Pantidou 1961 a & b, 1962 and 1964).

Requirements for formation of initials or primordia may be quite different to those conditions necessary for further development and expansion of these structures to form mature sporophores. Development may therefore represent two phases, the second developmental phase may have additional requirements for maturation of the hymenium and sporulation.

The requirements for the first phase may be largely nutritional and possibly directly triggered by factors impeding the development of hyphae at the mycelial front. This may be directly related to the ability of the hyphal tips of the lead hyphae to secure a minimum flow of nutrients to succeeding branch hyphae (Butler 1961).

Translocation of materials to the mycelial front where further hyphal development is prevented may result in accumulation by formation of initials or sclerotia or dissipation in formation of asexual reproductive structures in lower fungi. Where this process is prevented (possibly by adverse physical factors), autolysis with release of stored products into the medium may occur. The processes of primordia formation may be governed by, (a), the ability of the fungus to accumulate reserve materials from the substrate on which it is growing, this may be linked to the provision of a slowly metabolisable carbon source; (b), its ability to translocate these, which may be related to its ability to form mycelial

strands; (c), a low concentration of respiratory inhibitors, this may reflect a requirement for a certain low level of carbon dioxide; concentrations of this gas in excess of 0.67% cause inhibition of primordium development in Agaricus bisporus (Long and Jacobs 1974).

Removal of nutrients from the hyphal tips may be effected by colonisation of an inert substrate, removal by metabolism by associated microflora (Long 1968), or use of adsorbing agents such as activated charcoal. Depletion of nutrient status and inactivation may also result from low temperature treatment or mechanical injury.

Isolates forming primordia in pure culture may be those able to form reserve materials on laboratory media, possibly those producing poorest growth on these media (in terms of dry weight) or those less susceptible to inhibition of primordium formation with slightly elevated levels of carbon dioxide.

Physical factors such as light, temperature, humidity and aeration affect further fruitbody development (phase 2) and may determine degree of differentation. Light has been determined to be a requirement for pileus expansion of a wide range of basidiomycetes. A requirement for light was indicated for development of L. nuda sporophores though not determined conclusively.

Low temperature treatment may retard vegetative growth and stimulate primordium development, this has been shown to induce fruitbody development in Collybia velutipes (Kinugawa & Furukawa 1965). This may be an important mechanism in nature for L. nuda and L. saeva acting as a check to further vegetative development of surface hyphae; the accumulation of reserve materials over the previous months would account for sudden appearance of large numbers of sporophores.

ROLE OF BACTERIA IN FRUIT-BODY DEVELOPMENT

The presence of a large bacterial population within the 'cas^ing-layer' of the two phase system of Agaricus bisporus cultivation was first shown by Eger (1963) to be important in the induction of primordium formation. This was demonstrated by employing a divided Petri-plate system, the 'Halbschalen-test' which contained horizontally adjacent equal quantities of a mushroom growers compost (the 'vegetative' substrate) and a peat/chalk 'casing' material (the 'reproductive' support medium) within which there was development of a stimulatory bacterial population. A sterile casing material supported strong vegetative mycelial development and no primodium formation. The inclusion of granulated activated charcoal within the sterile medium promoted low numbers of primordia to develop.

This system represents the transition of a rich to a poor nutrient substrate in the upper section of the cased mushroom bed, though it cannot be considered to duplicate the gradient effects of moisture availability and carbon dioxide concentration which exist within the vertical system.

The work of Hayes, Randle and Last (1969) demonstrated that volatiles produced by the mushroom mycelium (Lockhard and Kneebone 1963) selectively favoured the development of a pseudomonad population comprising Pseudomonas putida and Pseudomonas Group IV. Ps. putida was subsequently shown to possess the ability to induce primordium development in agar culture by employing bacterial inoculation plugs placed at the periphery of a mycelial culture in a simple Petri-plate (single phase system) or on water-agar within a divided Petri-plate (Dual phase system - a refinement of the 'Halbschalen-test') (Hume and Hayes 1972; confirmed - Arrold 1972).

An ability of bacterial metabolites to stimulate primodium formation in A. bisporus was reported by Park and Agnihotri (1969a) However, in later experiments they recorded primoridal inducing responses under aseptic conditions from a range of vitamins (particularly biotin),

plant hormones (gibberellic acid), oxalic acid, autoclaved soil extracts and cell free bacterial filtrates. (Park and Agnihotri 1969b). Eger (1972) reports being unable to confirm the effect of biotin over various concentrations, under a range of septic and aseptic conditions.

The casing layer is considered (Eger 1972) to effectively shield an inducing microbial flora from the antibiotic effect of high concentrations of volatile inhibitor(s) produced by active A. bisporus mycelium, although close contact between inducing bacteria and individual or groups of hyphae was considered as requisit for primordium formation. In experiments using the 'Halbschalen-test' as a model system Pseudomonas putida was shown to only effectively inhibit the growth of certain mushroom strains, which was associated with an inducing effect, when compared with the action of a mixed bacterial casing flora, although the latter also produced variable growth inhibition within narrower limits. Pseudomonad cultures grown on acetone as sole carbon source (considered to be the only abundant volatile during the normal aerated conditions under which primordium formation occurs) when compared with controls lacking acetone, induced higher numbers of primordia. It was concluded that the volatile acetone may play a decisive role in supporting and maintaining a microflora which is stimulatory to primordium formation.

The induction of primordium differentiation and fruitbody development are processes which are dependent on the preliminary formation of an undifferentiated tissue mass, the 'initial'. The development of the 'initial' represents a fundamental and localised re-organisation in the pattern of hyphal branching and hyphal association, resulting in the multiplication and aggregation of hyphae to form a knot of tissue.

The development of the initial and the transition of the initial to the primordium are stages which are still ill-defined. It is probable that the development of the initial may be fairly readily induced by conditions of nutritional stress at the hyphal front, however, its further development into a primordium may be the critical stage which is dependant on the nutritional status of the colony and the existence of favourable external conditions. It may be that bacteria,

activated charcoal, or internal/external conditions causing autolysis, promote development of the initial, its further development may be in response to internal nutrient access though also external conditions of moisture, aeration, temperatureand light.

The formation of the initial and its further expansion and differentiation to form a primordium are therefore considered to be key stages for fruit-body development. These stages may be postulated to result as the culmination of key gene switches where metabolically active substances have acted to repress or derepress the expression of individual gene sequences, these substances arising externally (i.e. CO_2, antibiotics) or internally as a result of physical conditions (i.e. light, temperature, moisture stress).

The role of bacteria is uncertain, their effect has recently been successfully duplicated at fixed carbon dioxide concentrations (100 - 1000 p.p.m.) by the inclusion of activated charcoal in a sterile peat/chalk casing mix (Long and Jacobs 1974). Carbon dioxide is critical in controlling the transition of vegetative to reproductive hyphal growth.

The fruitbody inducing property of bacteria in natural soils and under commercial conditions has been explained by a number of plausible modes of action:

a) metabolism away of substances suppressing initiation produced by the lead hyphae, or the removal of nutrients leaking from the leading hyphae - this is possibly analagous to the action of activated charcoal;
b) the formation of antibiotics or other metabolites which act to suppress mycelial growth and induce development of initials - this may be correlated to the loss of inducing ability of bacterial isolates grown in pure culture over several generations (i.e. loss of an induced enzymic activity) (Eger 1968);
c) direct lysis of fungal hyphae - this has been observed for bacterial isolates by Eger (1968) on hyphae of Lepiota rhacodes and yeast cells.
d) the selected bacterial population may act to make available ferrous iron to the mushroom mycelium within the casing layer - a substantial proportion of the pseudomonad population which becomes established in

the casing layer is able to reduce ferric to ferrous iron (Hayes 1972, Hayes and Nair 1973).

Nutrition of fruitbody formation

A fruitbody inducing hormone has not been conclusively proven to exist; the work of Eger (1970) with Pleurotus has indicated that strongprimordial development can be stimulated by cultures developing from pieces of fruitbody tissue on biomalt agar or by the provision of relatively high levels of certain organic nitrogen sources (i.e. urea or asparagine). The work of Wessels (1967) with Schizophyllum commune has indicated that a constant supply of simple sugars is necessary for pileus development and expansion to occur within the developing primordium.

The processes of fruitbody formation in pure culture on agar media are frequently associated with autolysis coupled with exhaustion of medium components; this complex process renders analysis and comparison difficult, and may further be complicated by unknown conditions of aeration within Petri-plates and flask-culture systems.

Genetic Considerations

Genetical information and detailed information on the life history for these species is lacking.The inability of investigators to readily obtain the fruitbodies (sexual stage) in culture has no doubt accounted for their dismissal as suitable models for genetical studies.

All three species produce four spored basidia. Attempts at obtaining spore germination from spore prints of L.nuda and L. saeva deposited on the surface of malt agar slopes within MacCartney bottles were unsuccessful. All isolates of the species studied were obtained from cap tissue. Difficulties in obtaining germination of L.nuda spores were recorded by Norkrans (1950), however, Constantin and Matruchot (1898) report obtaining cultures of L.nuda from spores which germinated readily on spent tan bark and leaf substrates.

Isolates obtained by tissue culture are fertile dikaryons, this was indicated in these species by the possession of a regular distribution of clamp-connections at individual septa.

With the present knowledge possible mechanisms of out-breeding (bi- or tetrapolar mating type factors, the possession of multiple alleles of incompatibility loci) can only be the subject of speculation. Such information is likely to be compatible with that obtained for Coprinus and Schizophyllum. The use of isolates such as L.nuda (9) could further assist in clarifying these machanisms.

Heterokaryosis is suggested from nutritional data by the divergence between strains in ability to lyse cellulose and strength of the Bavendamm 'oxidase' reaction. Comparison of nutritional data for L.nuda isolates 7 and 8 for vitamin and trace metal requirement and also differential ability to utilise nitrate nitrogen by L.nuda and L.saeva strains is indicative of pronounced heterozygosity which is sexually maintained by mating type machanisms.

Commercial Exploitation

Commercial cultivation is potentially feasible for selected L.nuda strains providing that a means of inducing fruitbody development in a shorter period than three months is found. A cold temperature 'chilling' in specially constructed refrigeration rooms may facilitate the formation of 'timed' flashes of mushrooms. The advantages of cultivation of this species and L.saeva to that of A.bisporus is in an ability to utilise substrates requiring less nitrogenous supplementation and simpler preparation. The growth of single L.nuda and L.saeva isolates on a range of short composted substrates (table 13:1) indicates that good vegetative growth is produced at nitrogen levels of *c.* 1.6 g % N, stronger growth in non-peak heated substrates may indicate that these species favour short composted substrates.

It is probable that substrates comprising wood shavings or sawdust (preferably deciduous) or other sources of cellulosic/hemicellulosic materials such as straw, hay, cereal hulls or corn cobs could be

admixed with high grade nitrogen sources such as cotton-seed meal, soyabean meal or oat meal to produce suitable short composted substrates for commercial exploitation. The scheme envisaged would involve a preliminary mixing and presoaking of these materials over several days following which the material would be subjected to a short peak-heat (to induce a rapid thermophilic fermentation) and terminal pasteurisation.

It is probable that fruitbody yields are directly related to the quality of the nitrogen available, which may arise from microbial nitrogen or the nitrogenous supplements, and the presence of carbohydrate materials which can be readily but selectively utilised by these species such as microbially formed poly-saccharides or hemicelluloses and partly degraded cellulose. Ability to degrade lignin may be an important mechanism is gaining access to these materials by these species. These substrates should selectively favour the natural nutrition of these species the inclusion of a pasteurisation step would serve to eliminate competitors, rapid substrate colonisation should be attainable with a suitably chosen inoculum size.

Lepista nuda makes strong growth and is capable of producing abundant fruiting bodies in nature on partly rotted leaf substrates and therefore should be able to make good utilisation of the cereal straw. Tricholoma personatum as reported by Hard (1961) in America, 'delights to grow where an old sawmill has stood' and further more formed large numbers of fruiting bodies following growth on a compost of old green cobs remaining from a canning factory.

Good growth of L.nuda occurs at lower temperatures than required for A.bisporus, therefore, heating during substrate colonisation may not be necessary. Disadvantages include a longer incubation period required by this species, this disparity may be greatly reduced by substrate selection; and at present delayed fruitbody development. No information is available on yields, yield capacity will be directly related to substrate selection and preparation. Cooling may be required, however, this could be circumvented by cultivation during winter months, and selective use of low outside air temperatures.

A similar system may be employed for L. saeva, growth of this species is, however, less vigorous than that of L. nuda, this difficultly may to some degree be overcome by strain selection.

Lepista nuda has an attractive smell and taste for culinary purposes, it possesses a wide range of essential amino-acids (table 16:0). Mycelium obtained by submerged culture is reported to have a high food value in feeding trials (Bell, Erfle, Spencer & Reusser 1958). Lepista saeva is at present collected and sold in the Midland market towns of Burton-on-Trent, Loughborough, Leicester and Nottingham and is recorded as having been formally sold at Covent Garden market. Both species are sold in quantity in Continental open markets.

CONCLUSIONS

1. The present study formed an investigation into the physiology of three wild, edible, basidiomycete species: Lepista nuda (14 isolates), Lepista saeva (8 isolates), and Calocybe gambosa (5 isolates), including aspects of fruit-body formation. All isolates were obtained by cap-tissue culture. An attempt was made throughout the study to relate observations to the natural ecology of these species. A wholly saprophytic existence possibly as obligate rhizoplane inhabitants, excluding mycorrhizal association, was indicated from the data.

2. All species were routinely cultured and grew well on a malt extract agar, C.gambosa strains demonstrated progressively improved growth with adaptation to this medium during the course of the study. Good growth of L.nuda and L.saeva isolates was obtained on a formulated glucose-asparagine-salts plus thiamine basal liquid medium in stationary culture. Poor growth was shown by C.gambosa strains on this medium without the addition of certain growth factors. Growth on the synthetic medium by this species did not improve over the period of study.

3. Temperature optima of 20°C and 25°C were obtained for L.nuda isolates, 20°C for L.saeva, and 25°C for a single isolate of C.gambosa. Both L.nuda and L.saeva grew at low temperatures 10°C and 4°C (slightly). All species were inhibited at 30°C.

4. Optima in hydrogen ion concentration were determined at pH 7.0 for L.nuda, c.6.0 for L.saeva and 5.5 for C.gambosa. L.nuda exhibited a wider pH tolerance than L.saeva.

5. Investigation of trace metal requirements employing a range of ash additions to a minimal basal salt solution, calcium at relatively high levels of addition was found to be an important component of the complete synthetic medium for attaining high mycelial dry-weights. The probable importance of calcium as a metal-ion buffer is discussed.

6. All strains of L.nuda and L.saeva were heterotrophic for thiamine. Partial heterotrophy was indicated by some strains for biotin, pyridoxine and folic acid. C.gambosa grew slowly in the basal medium with thiamine addition, a partial heterotrophy for a broader range of vitamins by this species was indicated.

7. Optimal C:N ratios for vegetative growth were investigated employing the complete synthetic medium, by alteration of the level of carbon (glucose) maintaining nitrogen level (asparagine conc.,) constant. Growth efficiency (yield) was assessed for four series at increasing concentrations of nitrogen. An optimum C:N ratio of c.21:1 (and an extrapolated optimum nitrogen concentration of 0.1864gN/L) at 28 days incubation was determined for L.nuda and L.saeva. A somewhat higher C:N ratio was indicated as optimal for C.gambosa.

8. Single isolates of these three species were able to utilise a wide range of mono -, di -, tri - and polysaccharides. Combining carbon sources improved utilisation of some polysaccharides. Mannose, glucose, fructose, sucrose, maltose, raffinose, dextrin and starch were particularly favourable sources of carbon and energy.

9. Strains of all three species produced high yields on a broad range of organic, amide, and amino-acid sources of nitrogen. Degree of utilisation of ammonium and nitrate nitrogen was a variable character within species. Growth on these nitrogen sources was correlated with change in hydrogen ion concentration of the culture fluid. No strains tested utilised nitrite nitrogen.

10. A range of alcohols, simple organic acids and fatty acids did not produce significant ($P = 0.05$) yield increases in single L.nuda and L.saeva isolates when added as supplements (50,100 or 500 mg/L) to the complete synthetic medium. Improved yields by C.gambosa

following supplementation was produced by the rapid growth of sectors within the colony.

11. Inclusion of a range of leaf and manure extracts (at 0.5, 1.0 & 3.0 mg/L) into the basal medium produced highly significant growth increases (P = 0.001) of a single C.gambosa isolate in relation to the unsupplemented control value. This was particularly marked with Norway Spruce and European Larch leaf extracts. Significant growth promotion of L.nuda and L.saeva occurred with a considerably narrower range of extracts.

12. Assessment of cellulolytic ability by dissolution of finely milled cellulose fibres, and for polyphenol oxidase activity utilising the classical Bavendamm 'oxidase' test (discolouration of agars incorporating gallic or tannic acids by formation of coloured complexes) - this enzyme activity has been characterised as p-diphenol oxidase (Lindeberg 1946(b), 1948, 1950, Lindeberg et al., 1952(a & b)) - established that L.nuda and L.saeva are able to degrade cellulose and lignin, these enzyme activities were further shown to be a variable characteristic between isolates. C.gambosa demonstrated weak cellulase though high p-diphenol oxidase levels. These activities are discussed in relation to indirect evidence for possession of O-diphenol oxidase activity.

13. Growth rates of a range of L.nuda and L.saeva isolates were assessed through a variety of composted solid substrates in specially constructed cylindrical glass tubes. Extent of stranding was recorded in tubes and following colonisation of compost, peat/chalk casing in flasks. Development of compact nodules of tissue representing possibly fruit-body initials was recorded for four isolates of L.saeva. Strand development, nodulation and growth rate were used as criteria

for selection of strains for fruiting trials on a solid substrate. C.gambosa made poor growth or absence of growth into these composted substrates.

14. Lepista nuda strains forming fruit-bodies on a composted medium plus three strains of L.saeva which produced numerous tissue aggregates in tubes and flasks together with 3 C.gambosa strains, were assessed for their ability to form primordia on a natural agar medium under controlled conditions of lighting and temperature. A study was made of a single L.nuda strain which formed numerous primordia under this system of culture.

15. Fruit-body development by 3 L.nuda isolates was obtained on a commercial mushroom growers compost with a variety of leaf, soil and peat/chalk casing treatments, under artifical conditions. This culture system supported good colonisation of the compost and soil plus peat/chalk casing layers by 3 L.saeva isolates. However, fruit-body development of these isolates did not take place.

16. A diffusion-column technique was employed to assess the effect on colony growth-rate of a range of carbon dioxide and oxygen tensions, above and below atmospheric. Both L.nuda and L.saeva isolates were insensitive to partial pressures of oxygen, descending to 4.0% and below.

Strains of L.nuda, L.saeva and C.gambosa were sensitive to low partial pressures of carbon dioxide ($< 5.0\%$)

The ecological implications (as colonisers of surface leaf-litter and soils) of oxygen tolerance and intolerance to elevated levels of gaseous CO_2, is discussed.

ACKNOWLEDGEMENTS

I should like to thank Dr.W.A.Hayes for his supervision throughout this study; Dr.N.G.Nair, Department of Agriculture, Ryadalmere, N.S.W., for assistance with the gas-diffusion column; and Dr.R.Watling, Royal Botanic Gardens, Edinburgh, for encouragement and guidance, particularly concerning taxonomy of the Higher Fungi, and for provision of several isolates.

The assistance of the members of the Warwickshire Fungus Survey in obtaining isolates, and by Dr.G.M.Butler, Department of Botany, University of Birmingham, in co-ordinating these efforts, is gratefully acknowledged.

I should also like to thank postgraduate students within the Mushroom Science Laboratory, Richard Steane, Rosemarie Pearson, Nahida Haddad and Kenneth Jakeman (technician) and in the adjoining Fermentation Laboratory, Stephen Pannell and Robert Spensley, for companionship, assistance, discussion and criticism during the course of this study.

Appreciation is also extended for technical assistance provided by members of the Communications Media Department, Mr Johnston, Roger Banks and Roger Howes.

I would like, finally, to thank the Science Research Council for the postgraduate award, which made this study possible.

A P P E N D I X

APPENDIX: INDEX TO TABLES

TABLES 2.8 &9 (Lindeberg 1939) Phosphate Buffer Tables.

BUFFER CONCENTRATION		THEORETICAL pH.	EXPERIMENTALLY OBTAINED VALUES (of Lindeberg)
ml M/15 KH_2PO_4	ml N/10 HCl		
75	75	2.0	2.1
129	21	3.0	3.2
146	4	4.0	4.1
ml M/15 KH_2PO_4	ml M/15 Na_2HPO_4		
149.5	0.5	5.0	5.2
131.5	18.5	6.0	6.1
55	95	7.0	7.0
3	147	8.0	8.2
ml M/15 K_3PO_4	ml M/15 NaH_2PO_4		
13.5	136.5	9.0	8.8

Revised Buffer Table (of Modess 1941)

ml M/15 KH_2PO_4	ml N/10 HCl	THEORETICAL pH	Author's Values obtained with Glucose–Asparagine synthetic Medium
75	75	2.0	2.35
110	40	2.5	
130	20	3.0	3.1
140	10	3.5	
145	5	4.0	3.7
147	2.5	4.5	
149	0.5	5.0	4.32
ml M/15 KH_2PO_4	ml M/15 Na_2HPO_4		
145	5	5.5	
130	20	6.0	5.93
105	45	6.5	
60	90	7.0	6.9
20	130	7.5	
	150	7.8	7.73

TABLE 3.1-3 EFFECT OF TEMPERATURE ON GROWTH
Colony extension (cm) at 21 days incubation
(C.gambosa 21 & 42 days incubation).

TEMPERATURE (°C)	LEPISTA NUDA				LEPISTA SAEVA			CALOCYBE GAMBOSA	
	1	247.69	5	8	1	248.69	6	2	
4	0.8	0.8	1.6	1.5	1.4	1.4	1.3	0	0
10	1.7	3.5	4.7	4.3	4.9	4.4	4.6	1.2	1.6
15	3.7	4.5	6.5	5.9	6.7	5.9	5.8	2.9	6.3
20	5.7	5.8	7.0	6.5	8.5	6.5	6.6	3.9	6.8
25	7.0	6.8	6.2	4.7	7.3	5.3	6.0	4.8	8.5
30	0	0	0	0	0	0	0	0	0

TABLE 2.1-7 EFFECT OF HYDROGEN ION CONCENTRATION ON GROWTH (21 days incubation)

pH THEOR-ETICAL	pH ACTUAL	LEPISTA NUDA			LEPISTA SAEVA		CALOCYBE GAMBOSA	
		6 mg	7 mg	8 mg	248.69 mg	5 mg	6 mg	1 mg
3.0	3.1						22.0 (3.5)	
4.0	3.7	46.0 (5.22)		45.0 (4.9)			35.5 (5.65)	
4.5	4.32	73.2 (5.02)	38.1 (3.5)	60.0 (5.03)	48.0 (4.65)	35.1 (4.35)	52.7 (5.57)	24.2 (5.2)
5.0	5.0	85.0 (5.02)	117.0 (3.35)	84.0 (5.04)	154.9 (4.75)	83.6 (4.38)	87.6 (5.38)	40.7 (5.0)
6.0	5.93	103.0 (4.77)	138.5 (4.55)	82.0 (5.05)	223.0 (5.6)	183.7 (5.1)	74.3 (5.9)	18.2 (5.85)
7.0	6.9	108.1 (5.72)	190.5 (5.45)	97.5 (5.4)	124.0 (6.75)	102.0 (6.56)	45.1 (6.1)	26.0 (7.08)
8.0	7.73	75.6 (7.3)	80.5 (6.6)	64.0 (7.0)	83.0 (7.3)	50.0 (5.65)	39.0 (6.68)	

( ) pH culture fluid, post incubation.

TABLE 4.1—2

ASH OR METAL ION ADDITION	Lepista nuda		Lepista saeva	
	7 mg	8 mg	6B mg	5 mg
BASAL SOLUTION	93.8 (4.75)	119.3 (5.1)	42.6 (5.8)	44.0 (4.75)
BASAL + BEECH LEAF LITTER ASH	143.7* (6.0)	133.2 (5.75)	92.5** (6.05)	139.6 (6.0)
BASAL + CHESTNUT LEAF LITTER ASH	166.5** (5.85)	95.7 (6.1)	113.0** (5.95)	132.8** (5.45)
BASAL + LARCH LEAF LITTER ASH	166.6** (5.45)	100.1 (5.8)	154.0** (5.7)	122.3** (4.75)
BASAL + HORSE MANURE ASH	194.5** (5.75)	110.6 (5.95)	115.9** (5.7)	116.8** (5.2)
BASAL + MALT EXTRACT ASH	158.6** (5.7)	88.5 (6.15)	63.6* (5.6)	115.8** (5.3)
BASAL + YEAST EXTRACT ASH	147.9* (5.4)	143.9 (5.1)	127.7** (5.9)	63.7* (4.55)
BASAL + SYNTHETIC MINERAL SOLUTION	63.5 (4.45)	141.6 (5.5)	83.6** (5.6)	40.2 (4.6)
BASAL + ZINC	62.8 (4.35)	115.7 (5.1)	64.3* (5.75)	37.4 (4.4)
BASAL + ZINC + CALCIUM	87.1 (4.4)	89.4 (5.2)	98.8** (5.15)	50.3 (4.3)
COMPLETE SYNTHETIC MEDIUM	208.7** (4.9)	108.5 (5.4)	156.4** (5.8)	158.7** (4.95)

* SIGNIFICANT (P = 0.01)

** SIGNIFICANT (P = 0.001)

Increase in yield to that attained on Basal solution.

( ) pH value of culture fluid, post-incubation.

TABLE 5.1-3

VITAMIN SUPPLEMENT	LEPISTA 7 mg	NUDA 8 mg	LEPISTA 5 mg	SAEVA 6B mg	CALCOCYBE 1 mg	GAMBOSA 2 mg
YEAST EXTRACT	146.6	101.2	148.0	189.4	10.3	12.0
COMPLETE VITAMIN SOLN; (CV)	133.7	76.4	121.0	124.8	3.6	4.0
CV - PYRIDOXINE	99.4*	68.0	91.5*	85.3*	0.8	1.0
CV - THIAMINE	60.8*	44.3*	39.1*	27.2*	0.6	0.7
CV - RIBOFLAVIN	133.3	94.6	107.3	111.0	1.0	4.4
CV - CALCIUM PANTOTHENATE	124.6	84.9	121.0	99.2*	0.6	1.2
CV - NIACIN	122.2	83.3	121.7	95.9*	0.6	0.6
CV - BIOTIN	107.*	79.7	86.4*	79.6*	0.7	0.6
CV - p - AMINO-BENZOIC ACID	105.9*	83.5	103.6	94.6*	1.0	0.7
CV - FOLIC ACID	92.7*	97.0	86.0*	86.2*	0.8	0.6
VITAMIN - FREE CONTROL	52.3*	37.9*	26.1*	20.7*	1.7	2.7

* Significantly lower (P = 0.001) than yield on complete Vitamin solution.

TABLE 6.1 EFFECT OF C/N RATIO ON GROWTH

CALOCYBE GAMBOSA (28 days incubation)

C/N RATIO	MYCELIAL YIELD (^{mg}/Dry Wt) NITROGEN LEVEL (gN/L)			TERMINAL pH NITROGEN LEVEL (gN/L)		
	0.0932	0.3728	0.5592	0.0932	0.3728	0.5592
9:1	17.2	20.0	25.7	6.25	6.1	6.0
12:1	20.1	23.8	33.0	6.15	6.4	6.0
15:1	21.5	26.4	35.9	6.18	6.02	5.7
18:1	22.8	27.4	38.0	6.31	6.05	5.3
21:1	24.0	28.1	39.0	6.22	5.8	5.3
25:1	24.6	34.1	41.8	6.22	5.85	5.05
28:1	25.9	35.9	44.2	6.25	5.8	4.7

TABLE 6.0 PROPORTIONS BY WEIGHT OF GLUCOSE AND ASPARAGINE COMPONENTS AT INDIVIDUAL C/N RATIOS

1g GLUCOSE = 0.4g CARBON 1g ASPARAGINE = 0.32g CARBON ($NH_2CO.CH_2.CH(NH_2)(COOH.H_2O)$)
0.1864g NITROGEN

C/N RATIO	NITROGEN LEVEL (g N/LITRE)			
	0.0932	0.1864	0.3728	0.5592
	GRAMS GLUCOSE PER LITRE			
9:1	2.1	4.2	8.4	12.6
12:1	2.8	5.6	11.2	16.8
15:1	3.5	7.0	14.0	21.0
18:1	4.2	8.4	16.8	25.2
21:1	4.9	9.8	19.6	29.4
25:1	5.9	11.7	23.4	35.1
28:1	6.5	13.0	26.0	39.0

TABLE 6.2 EFFECT OF C/N RATIO ON GROWTH

LEPISTA NUDA 7 (28 days incubation)

C/N RATIO	MYCELIAL YIELD ($^{mg}/_{Dry\ Wt}$) NITROGEN LEVEL (gN/L)				TERMINAL pH NITROGEN LEVEL (gN/L)			
	0.0932	0.1864	0.3728	0.5592	0.0932	0.1864	0.3728	0.5592
9:1	30.9	68.8	121.5	191.0	6.15	6.2	5.95	7.1
12:1	46.4	76.6	118.1	138.6	5.8	5.7	4.35	4.4
15:1	63.2	84.7	110.7	109.9	5.6	5.25	4.0	3.82
18:1	68.8	104.5	120.1	129.1	5.65	4.8	3.8	3.8
21:1	77.3	114.2	139.6	133.5	5.4	4.45	3.75	3.8
25:1	94.7	127.6	157.4	150.7	4.95	3.85	3.5	4.0
28:1	101.1	81.5	165.4	154.0	4.8	3.5	3.85	4.2

LEPISTA SAEVA 6A

9:1	43.6	75.6	162.6	246.8	6.3	6.7	6.7	6.6
12:1	61.6	117.9	190.5	277.6	6.1	6.32	5.2	5.68
15:1	78.0	142.6	211.8	305.3	5.95	5.93	4.05	4.15
18:1	88.1	162.0	288.0	321.5	5.9	5.8	4.1	4.25
21:1	101.7	193.8	303.5	330.4	5.9	5.6	3.9	3.9
25:1	122.3	147.3	285.3	367.9	5.65	4.1	3.85	4.2
28:1	120.8	136.5	244.9	280.4	5.05	3.93	3.8	4.2

TABLES 6.3-4 EFFECT OF C/N RATIO ON GROWTH

LEPISTA NUDA 7.

0,1864 gN/L SERIES

C/N RATIO	g GLUCOSE PER LITRE	MYCELIAL YIELD (mg/DryWt) DAYS (OF HARVEST) 7	14	21	28	TERMINAL pH DAYS (OF HARVEST) 7	14	21	28
9:1	4.2	21.4	24.9	29.4	38.1	5.92	5.92	5.9	5.3
12:1	5.6	23.1	32.0	37.6	47.9	5.92	5.85	5.6	5.05
15:1	7.0	21.5	31.0	43.6	59.9	5.93	5.82	5.5	4.9
18:1	8.4	26.5	35.8	46.6	62.4	5.9	5.75	5.6	5.3
21:1	9.8	26.0	36.4	52.5	77.0	5.9	5.7	5.53	5.13
25.:1	11.7	26.5	41.9	54.7	71.0	5.9	5.8	5.58	5.2
28:1	13.0	31.4	44.6	62.5	69.0	5.9	5.73	5.55	5.1

0.5592 gN/L SERIES

9:1	12.6	30.7	55.5	74.5	62.1	5.9	5.73	5.8	5.65
12:1	16.8	37.5	65.2	80.4	99.6	5.9	5.6	5.7	5.97
15:1	21.0	44.2	65.2	101.5	116.9	5.9	5.7	5.83	5.96
18:1	25.2	44.7	85.0	103.8	136.0	5.9	5.5	5.75	5.87
21:1	29.4	51.5	94.7	117.5	141.0	5.82	5.45	5.55	5.87
25:1	35.1	51.9	78.8	103.2	149.0	5.8	5.68	5.5	5.76
28:1	39.0	49.6	67.2	93.6	171.0	5.8	5.65	5.55	5.7

TABLES 6.5-6 EFFECT OF C/N RATIO ON GROWTH

LEPISTA SAEVA 6B

0.1864 gN/L SERIES

C/N RATIO	g GLUCOSE PER LITRE	MYCELIAL YIELD (mg/DryWt) DAYS (OF HARVEST) 7	14	21	28	TERMINAL pH DAYS (OF HARVEST) 7	14	21	28
9:1	4.2	25.2	51.6	82.4	80.4	6.0	5.95	6.0	6.7
12:1	5.6	24.5	64.7	103.8	107.6	5.97	5.95	6.0	6.45
15:1	7.0	27.5	74.3	124.7	127.0	6.0	5.9	5.95	6.1
18:1	8.4	27.2	71.3	133.8	147.0	6.0	5.9	5.7	5.8
21:1	9.8	28.2	73.8	142.4	167.1	5.95	5.9	5.42	5.64
25:1	11.7	26.1	88.7	146.3	160.8	5.95	5.88	5.6	4.65
28:1	13.0	23.2	65.9	112.3	110.0	5.95	5.85	5.75	3.5

0.5592 gN/L SERIES

9:1	12.6	23.9	72.2	140.8	208.6	5.95	5.76	4.9	5.75
12:1	16.8	25.8	81.7	167.9	228.0	5.95	5.7	4.55	3.75
15:1	21.0	31.4	101.1	184.6	246.8	5.95	5.72	5.1	3.9
18:1	25.2	33.0	100.4	190.2	254.0	5.9	5.65	4.95	4.12
21:1	29.4	35.0	100.0	191.4	263.0	5.85	5.65	5.25	4.1
25:1	35.1	34.5	101.9	219.4	284.0	5.8	5.62	5.21	4.18
28:1	39.0	32.5	98.7	200.0	275.0	5.75	5.62	5.3	4.35

TABLE 7.1–3

CARBON SOURCES	LEPISTA NUDA 7 mg	LEPISTA SAEVA 6B mg	CALOCYBE GAMBOSA 2 mg
D – XYLOSE	4.3	2.0	14.5
D – RIBOSE	28.7	50.9	6.8
L – ARABINOSE	3.9	2.4	9.6
L – SORBOSE	2.2	0.9	3.5
D – GLUCOSE	212.7	345.0	18.2
D – FRUCTOSE	198.8	302.0	19.8
D – MANNOSE	203.5	342.5	18.0
D – GALACTOSE	97.8	20.2	17.8
SUCROSE	243.5	307.6	12.6
MALTOSE	244.9	269.3	20.9
CELLOBIOSE	188.5	264.1	16.3
LACTOSE	90.5	85.3	13.5
TREHALOSE	224.2	223.7	10.3
D – MELIBIOSE	46.2		
RAFFINOSE	263.3	31.1	11.9
DEXTRIN	286.0	308.8	7.5
STARCH	160.2	296.4	9.3
GLYCOGEN	201.0	278.0	7.4
PECTIN	29.2	1.5	6.7
INULIN	120.4	240.2	14.8
XYLAN	89.7	251.1	7.2
∝- CELLULOSE	80.3	85.0	5.9
L – RHAMNOSE	15.4	9.8	4.5
D – DULCITOL	42.7	10.1	7.5
D – MANNITOL	81.4	13.4	12.0
D – SORBITOL	136.8	68.7	

TABLE 7.1-3 cont:

CARBON SOURCES	LEPISTA NUDA 7 mg	LEPISTA SAEVA 6B mg	CALOCYBE GAMBOSA 2 mg
GALACTOURONIC ACID	24.1	24.1	3.4
CITRIC ACID	4.7	1.5	3.1
DL - MALIC ACID	5.2	6.9	3.2
D - TARTARIC ACID	7.7	4.0	3.7
SUCCINIC ACID	11.0	10.1	3.0
CALCIUM OXALATE	3.3	0.2	1.2
CONTROL	3.6	4.4	3.3
L.S.D. (Inclusive of combined carbon sources)	21.6	17.7	2.7

TABLE 8.1-3

NITROGEN SOURCES	LEPISTA 7 mg	NUDA 8 mg	LEPISTA 6B mg	SAEVA 5 mg	CALOCYBE 1 mg	GAMBOSA 2 mg
PEPTONE	169.1 (4.75)	188.8 (4.95)	106.4 (4.9)	122.1 (4.65)	9.7 (5.55)	18.7 (5.75)
CASEIN HYDROLYSATE	174.1 (4.55)	140.4 (5.4	158.8 (5.65)	214.5 (5.2)	25.7 (5.95)	15.7 (5.9)
UREA	197.6 (6.6)	155.6 (6.85)	61.3 (7.15)	81.8 (6.95)	4.4 (7.65)	1.6 (7.6)
ASPARAGINE	208.7 (4.9)	108.9 (5.4)	156.4 (5.8)	158.7 (4.95)	19.3 (6.25)	1.9 (6.2)
PROPIONAMIDE	60.6 (5.6)	92.2 (6.9)	54.7 (6.0)	79.2 (6.45)	1.2 (5.95)	3.6 (5.95)
AMMONIUM BICARBONATE	178.6 (4.25)	150.2 (6.25)	128.3 (6.5)	110.6 (4.7)	1.4 (7.35)	1.9 (7.45)
AMMONIUM TARTRATE	139.7 (4.55)	82.2 (4.9)	69.9 (4.9)	74.3 (4.6)	9.5 (5.5)	6.4 (5.85)
SODIUM NITRATE	153.7 (5.7)	66.9 (5.65)	24.3 (5.95)	133.0 (6.55)	4.1 (5.8)	2.3 (5.85)
SODIUM NITRITE	1.3 (6.0)	1.2 (6.05)	0.2 (6.1)	1.7 (6.05)	0.7 (6.1)	1.0 (6.1)
POTASSIUM NITRATE	205.5 (5.95)	61.2 (5.75)	35.2 (6.15)	141.4 (6.8)	7.8 (5.8)	3.8 (5.9)
CONTROL	4.5 (5.5)	4.1 (6.0)	0.7 (5.9)	3.5 (5.85)	3.6 (5.9)	1.7 (5.95)

( ) pH value of culture fluid, post incubation.

TABLE 8.4-6

AMINO-ACIDS	LEPISTA NUDA 7 mg	LEPISTA SAEVA 6B mg	CALOCYBE GAMBOSA 2 mg
GLYCINE	212.6 (4.3)	245.2 (5.2)	4.1 (5.65)
DL - ∝ ALANINE	207.9 (4.8)	170.0 (5.5)	4.4 (5.55)
DL - VALINE	165.8 (4.3)	44.1 (5.1)	11.1 (5.23)
L - LEUCINE (+ISOLEUCINE)	220.6 (3.7)	80.6 (4.1)	2.5 (5.85)
L - CYTEINE	42.7 (4.58)	31.9 (5.4)	3.4 (5.41)
L - CYSTINE	29.9 (5.18)	12.6 (4.2)	5.7 (5.83)
DL - METHIONINE	99.7 (4.3)	29.6 (4.0)	6.5 (5.55)
L - ASPARTIC ACID	270.5 (5.55)	222.2 (7.2)	5.6 (5.94)
L - GLUTAMIC ACID	193.6 (6.55)	243.2 (7.48)	6.3 (5.74)
L - LYSINE - HCL	3.1 (5.5)	3.6 (5.65)	2.8 (5.75)
L - SERINE	188.8 (4.6)	247.6 (4.6)	5.8 (5.5)
L - THREONINE	125.8 (4.92)	39.7 (4.95)	5.2 (5.75)
L - ARGININE	156.8 (3.62)	73.2 (3.65)	5.4 (4.62)
L - HISTIDINE	3.8 (5.8)	1.6 (5.8)	3.0 (5.92)
DL-β-PHENYLALANINE	7.3 (5.18)	12.1 (4.6)	9.0 (5.8)
L - TYROSINE	11.0 (5.6)	1.4 (5.7)	4.7 (5.75)
L - TRYPTOPHAN	17.4 (4.9)	22.3 (5.34)	5.8 (5.6)
L O PROLINE	2.0 (5.7)	1.2 (5.85)	3.5 (5.9)
L - HYDROXY-PROLINE	0.8 (5.68)	0.2 (5.85)	4.6 (5.9)
ASPARAGINE	212.8 (4.2)	335.6 (4.94)	4.3 (5.43)
GLUTAMINE	193.2 (3.47)	67.3 (3.7)	6.7 (5.13)
AMINO-ACID MIX	170.2 (4.07)	71.8 (4.08)	17.6 (5.37)
CASEIN HYDROLYSATE	216.8 (3.95)	313.6 (5.2)	11.3 (5.35)
CONTROL	1.6 (5.82)	0.7 (5.88)	5.1 (5.9)

( ) pH culture fluid, post incubation.

TABLE 10.1

EXTRACTED MATERIAL	EXTRACT CONCENT-RATION (mg/ml)	LEPISTA NUDA 7 mg	LEPISTA SAEVA 6B mg	CALOCYBE GAMBOSA 2 mg
PIG MANURE	0.5	164.8	248.5	28.3
	1.0	237.8*	200.0	32.3
	3.0	232.8	179.7	31.3
BULLOCK MANURE	0.5	238.8*	237.7	47.8**
	1.0	247.0*	251.2	36.6
	3.0	279.0**	236.0	48.1**
HORSE MANURE	0.5	172.8	241.3	35.5
	1.0	203.2	230.8	79.6**
	3.0	233.6	172.0	102.8**
S. CHESTNUT LEAVES	0.5	207.3	247.0	51.0**
	1.0	177.2	282.0**	46.0**
	3.0	197.0	239.3	41.8*
BEECH LEAVES	0.5	254.5**	258.0	95.3**
	1.0	225.3	272.3*	39.3*
	3.0	277.9**	184.9	36.5
S. BIRCH LEAVES	0.5	225.9	86.7	39.1*
	1.0	263.7**	121.5	51.6**
	3.0	288.0**	147.2	40.0*
OAK LEAVES	0.5	222.0	164.2	46.2**
	1.0	263.5**	164.3	45.1**
	3.0	212.0	202.5	60.6**
C. BEECH LEAVES	0.5	194.4	175.0	45.3**
	1.0	144.6	209.4	42.3*
	3.0	61.8	82.8	7.2
L. CYPRESS LEAVES	0.5	211.8	231.8	44.5**
	1.0	204.5	207.8	36.8
	3.0	179.0	189.7	28.2
PINE LEAVES	0.5	148.6	131.0	41.5*
	1.0	147.0	131.0	37.2
	3.0	142.5	133.0	52.0**
SPRUCE LEAVES	0.5	154.2	233.7	128.7**
	1.0	196.9	220.7	118.3**
	3.0	235.8	171.5	126.0**
LARCH LEAVES	0.5	157.0	279.1*	107.0**
	1.0	218.4	275.3*	104.0**
	3.0	177.6	227.5	113.0**
CONTROL		199.2	213.9	17.9

* SIGNIFICANT (P=0.01)
** SIGNIFICANT (P=0.001)
} INCREASE IN YIELD TO THAT ATTAINED ON THE CONTROL.

TABLE 15.1-2 EFFECT OF OXYGEN CONCENTRATION ON COLONY GROWTH RATE.

0 - 21.0% OXYGEN.

OXYGEN CONCENTRATION %	LEPISTA NUDA 7 mm/day	LEPISTA SAEVA 5 mm/day	LEPISTA NUDA 247.69 mm/day	LEPISTA SAEVA 248.69 mm/day
0.30	0.42	0.32	0.3	0.3
1.25	3.39	1.79	2.11	1.41
1.76	3.54	1.88	2.22	1.44
2.27	4.10	1.93	2.27	1.56
2.79	4.16	2.16	2.20	1.62
3.77	4.31	2.19	2.13	1.67
4.73	4.46	2.26	2.06	1.77
5.75	4.42	2.24	2.24	1.76
6.75	4.63	2.25	2.16	1.70
7.76	4.56	2.35	2.18	1.75
8.75	4.56	2.32	2.11	1.95
10.72	4.71	2.32	2.24	2.10
12.69	4.60	2.28	2.20	1.95
14.69	4.63	2.35	2.34	2.14
16.69	4.54	2.37	2.30	2.10
18.69	4.64	2.37	2.24	2.06
21.00	4.50	2.41	2.14	2.05

TABLE 15.3 EFFECT OF CARBON DIOXIDE CONCENTRATION ON COLONY GROWTH RATE.

0 - 12.0% CO_2

CARBON DIOXIDE CONCENTRATION %	LEPISTA NUDA 247.69 mm/day	LEPISTA SAEVA 248.69 mm/day
0.69	1.91	1.52
1.06	2.06	1.50
1.43	1.76	1.50
1.81	1.77	1.38
2.51	1.80	1.35
3.21	1.64	1.30
3.95	1.67	1.24
4.69	1.60	1.20
5.41	1.67	1.07
6.12	1.65	1.06
7.55	1.32	1.07
8.98	1.03	1.02
10.43	0.77	0.82
11.87	0.83	0.40

TABLE 15.4-5 EFFECT OF CARBON DIOXIDE CONCENTRATION ON COLONY GROWTH RATE.

0 - 5% CO_2

CARBON DIOXIDE CONCENTRATION %	LEPISTA NUDA 7 mm/day	LEPISTA SAEVA 6B mm/day	LEPISTA SAEVA 5 mm/day	CALOCYBE GAMBOSA 1 mm/day
0	4.52	2.10	2.2	1.44
0.23	4.72	2.60	2.2	1.24
0.35	4.72	2.60	2.1	0.79
0.48	4.60	2.60	2.1	0.76
0.60	4.40	2.56	2.1	0.68
0.84	4.20	2.44	2.1	0.68
1.07	3.96	2.40	2.1	0.65
1.32	3.56	2.28	1.89	0.65
1.56	3.48	2.16	1.89	0.65
1.80	3.00	2.00	1.89	0.62
2.04	3.00	1.96	1.72	0.62
2.52	2.24	1.80	1.61	0.60
3.00	1.56	1.68	1.5	0.41
3.48	1.52	1.60	1.5	0.35
3.96	1.12	1.52	1.4	0.35
4.49	1.10	1.40	1.16	0.35
5.00	0.92	1.32	1.16	0.32

Figure 15.6 Calibration for Carbon Dioxide concentration gradient within Diffusion-column, adjusted for values of 1,0% & 0% within the individual displacement chambers (readings obtained with Fisons Mini Gas-Chromatograph)

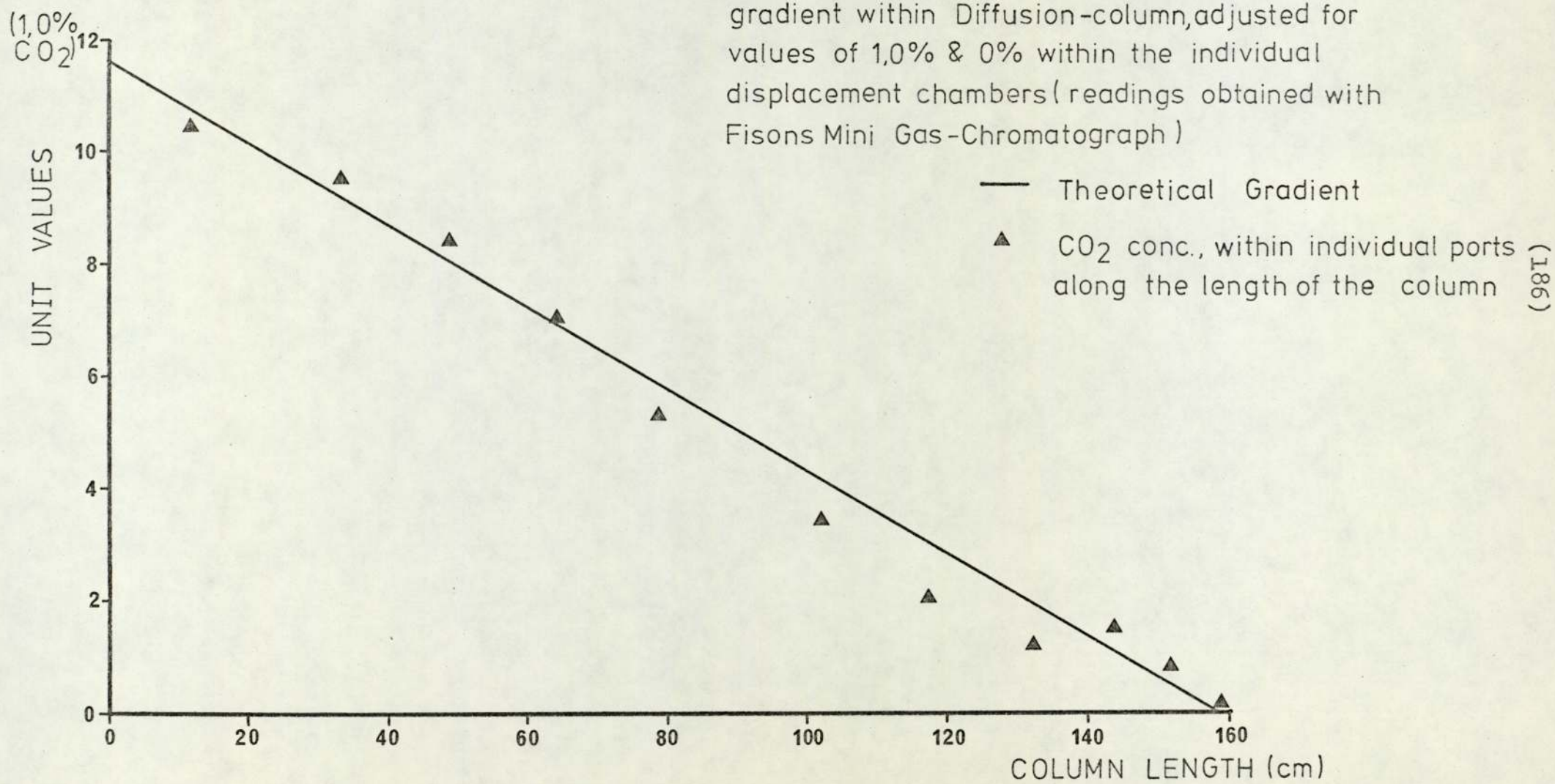


TABLE 16.0

AMINO-ACID ANALYSIS *Lepista nuda* SPOROPHORES †

AMINO-ACID	ISOLATE 8		ISOLATE 9	
	% DRY-WEIGHT	% TOTAL AMINO-ACIDS	% DRY-WEIGHT	% TOTAL AMINO-ACIDS
GLUTAMIC ACID	3.58	17.9	4.23	19.7
ASPAR TIC ACID	1.98	9.9	2.36	11.0
ALANINE	1.73	8.6	1.73	8.1
LEUCINE	1.4	7.0		
THREONINE	1.39	6.9	1.55	7.2
ARGININE	1.30	6.5	1.45	6.8
SERINE	1.28	6.4	1.38	6.4
VALINE	1.21	6.0	1.31	6.1
LEUCINE			1.17	5.4
LYSINE	1.20	6.0	1.11	5.2
GLYCINE	1.02	5.1	1.11	5.2
PROLINE	0.99	4.9	1.09	5.1
PHENLYALANINE	0.97	4.8	0.95	4.4
ISOLEUCINE	0.90	4.5	0.95	4.4
HISTIDINE	0.49	2.4	0.53	2.5
TYROSINE	0.39	1.9	0.39	1.8
METHIONINE	0.22	1.1	0.17	0.8
TRYPTOPHAN	*		*	
CYSTINE	*		*	
TOTAL	20.1		21.5	

* DESTROYED DURING ANALYSIS

† SPOROPHORES HARVESTED AS 'FLATS'.

TABLE 17.0

British Oxygen Co., Ltd., Normal Product specification for Commercial Compressed Oxygen, Nitrogen and Carbon Dioxide.

1. OXYGEN

Oxygen	99.7% (on dry gas)
Nitrogen) Argon)	< 3000 v.p.m.
Carbon Dioxide	< 2 v.p.m.
Carbon Monoxide	< 1 v.p.m.
Hydrocarbons as CH_4	< 20 v.p.m.
Water Vapour	< 50 v.p.m.

2. NITROGEN

Nitrogen	99.7%
Oxygen	< 3000 v.p.m.
Carbon Dioxide	< 1 v.p.m.
Carbon Monoxide	< 1 v.p.m.
Hydrocarbons as CH_4	< 1 v.p.m.
Argon	included in Nitrogen
Moisture	< 200 v.p.m.

3. CARBON DIOXIDE

Inert Gases (insol. in KOH soln.,)	100 pp.m v/v
Hydrocarbons as CH_4	10 pp.m w/w
Oil	2 pp.m w/w
Sulphur (inorganic)	0.5p.p.m w/w
Halogens	1 p.p.m w/w
Moisture	10 p.p.m w/w

TABLE 18.1 (A&B) Natural/Semi-synthetic Media.

A. Modified Cellulose Medium (employed in Petri-plates).

L- asparagine	1g.
Cellulose (4%) suspension	250 ml.
(72HR BALL-MILLED WHATMAN CFII)	
M/15 KH_2PO_4	131.5 } pH 6.0
M/15 Na_2HPO_4	18.5 }
$MgSO_4.7H_2O$	0.5g
Ferric Citrate	5 mg.
(1% soln. in Citric Acid)	
NaCl	0.1g
$ZnSO_4.7H_2O$	4.4mg.
$CaCl_2.2H_2O$	0.1g
'Start glucose'	50mg.
Oxoid Yeast Extract	50mg.
Distilled Water	to 1000 ml.
Oxoid Agar No.3	15g.

B. Cellulose Agar (Eggins & Pugh 1962) (employed in cellulose columns).

L - asparagine	0.5g.
Cellulose suspension (4%)	250 ml.
KH_2PO_4	1g.
$(NH_4)_2SO_4$	0.5g.
KCl	0.5g.
$MgSO_4.7H_2O$	0.2g.
$CaCl_2.2H_2O$	0.1g.
Oxoid Yeast Extract	0.5g.
Distilled Water	to 1000 ml.
Oxoid Agar No.3	20g.

TABLE 18.2 (A B&D) Natural/Semi-synthetic Media.

A. Lutz Malt Agar (Singer 1962).

Malt Extract (Boots Pure Drug Co.)	10g.
NH_4NO_3	1g.
$NH_4H_2PO_4$	1g.
$MgSO_4.7H_2O$	0.1g
Ferric Sulphate	0.1g.
$MnSO_4.7H_2O$	0.05g
Distilled water	to 1000 ml.
Oxoid Agar No.3	15g.

B. Treschow Wort Agar.(Treschow 1944).

Glucose	10g.
L-asparagine	1g.
KCl	0.2g.
$MgSO_4.7H_2O$	0.2g.
$CaCl_2.2H_2O$	0.2g.
$FeCl_3.6H_2O$ (1% soln.)	1 ml.
M/15 Na_2HPO_4	75 ml.
M/15 KH_2PO_4	15 ml.
12^o Wort	10 ml.
(pH adjusted to 6.8)	
Distilled water	900 ml.
Oxoid Agar No.3	15g.

Precautions to avoid precipitation of insoluble salt complexes involve adding calcium and ferric chlorides plus phosphates separately to a cooled medium.

D. COMPOSITION OF PEAT/CHALK 'CASING' MIXTURE.

	$V/_V$	$W/_W$
Ground Chalk	1	1
Medium Grade Sphagnum Peat	18	2.3
Water	8	5

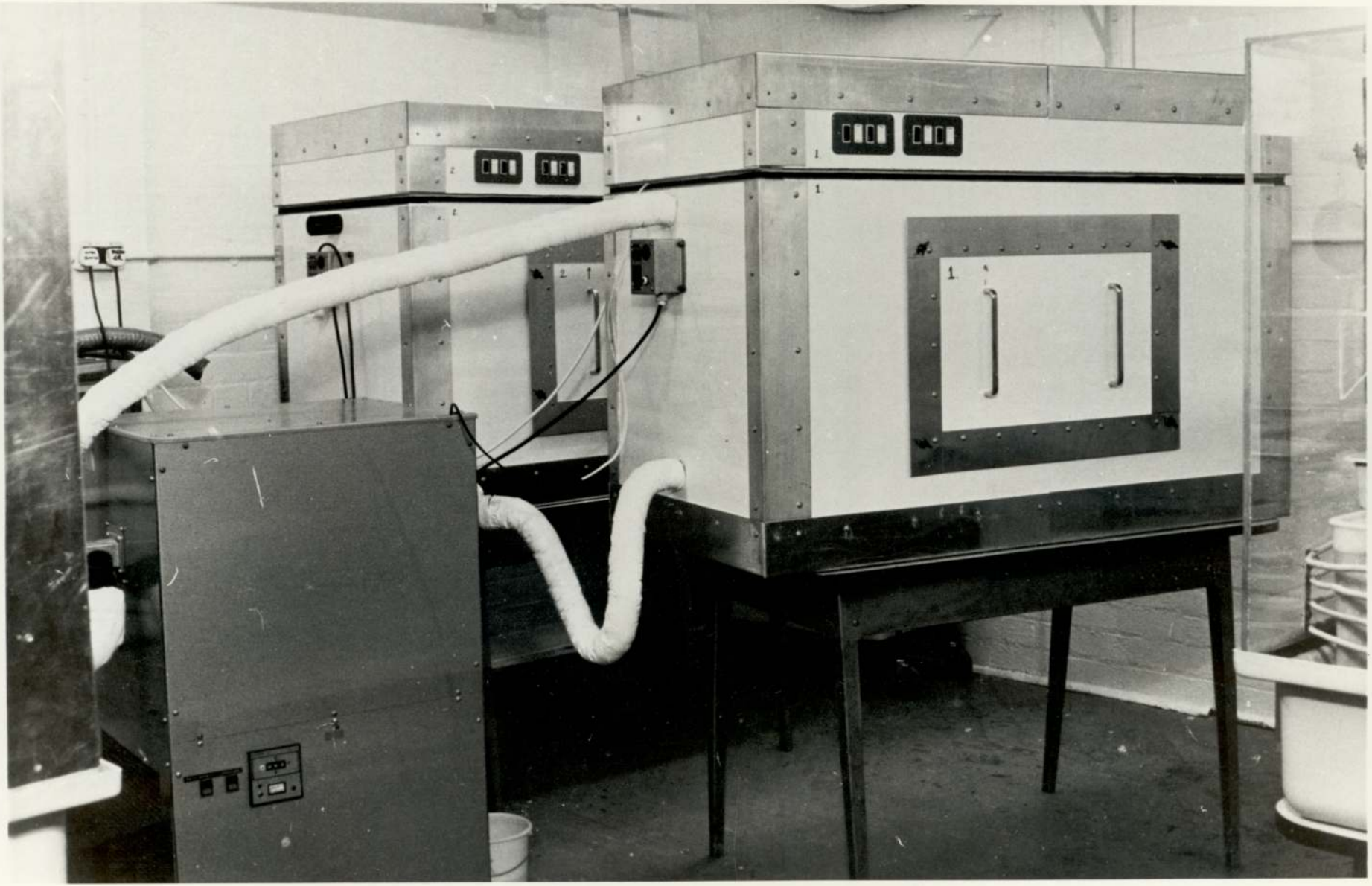
TABLE 18.2 (c) Natural/Semi-synthetic Media.

C. Hagem's Malt Agar (modified after Modess 1941).

Malt Extract (Boots Pure Drug Co.)	5g.
Glucose	5g.
KH_2PO_4	0.5g.
$MgSO_4.7H_2O$	0.5g.
NH_4Cl.	0.5g.
$FeCl_3.6H_2O$ (1% soln).	5mg.
Distilled Water	to 1000 ml.
Oxoid Agar No.3	15g.

P L A T E 29.

ILLUMINATED COOLED CABINETS.



ILLUMINATED COOLED CABINETS (FIGURES 16.0 A, B & C)

KEY

LIGHTING HOOD

A. Rocker switches and neons

B. 4 4′ 'daylight' fluorescent tubes

C. Capacitors and ballast

D. Sealed clear perspex partition

MAIN CABINET. EXTERNAL FEATURES

E. Foam-rubber seal

F. 'Humex' thermostat and temperature sensor

G. Air inlet and outlet

H. Sealed sliding inner perspex door

I. Cabinet door (push-fit)

J. Rivited right angle aluminium frame

MAIN CABINET. INTERNAL FEATURES

K. Plastic tubing serving as air-ducting

L. Polypropylene-glassfibre ('Celmar')/Expanded polyurethane/Epoxy-resin Laminate

M. Polypropylene welded seams

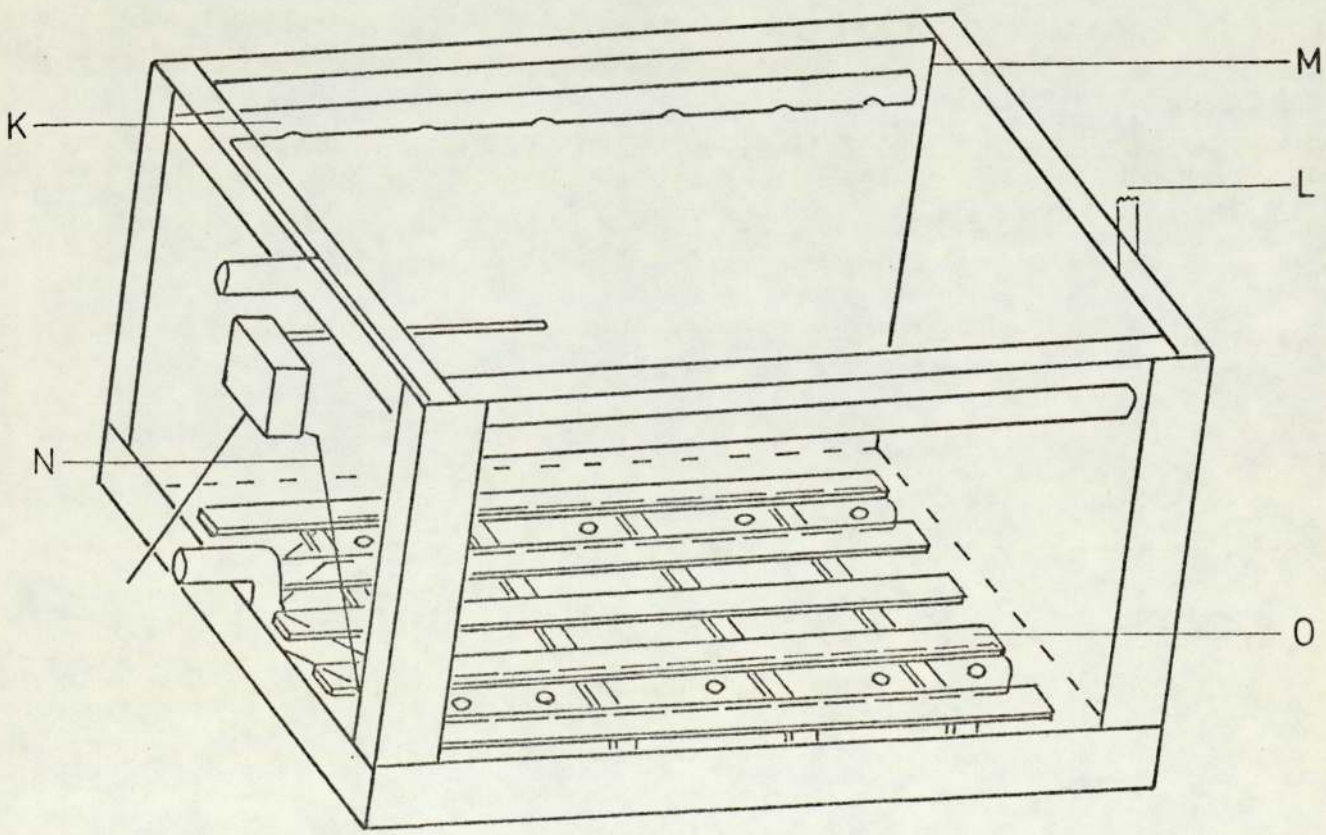
N. 'Humex' air-warming cable fitted beneath wooden support rack

O. Wooden support rack

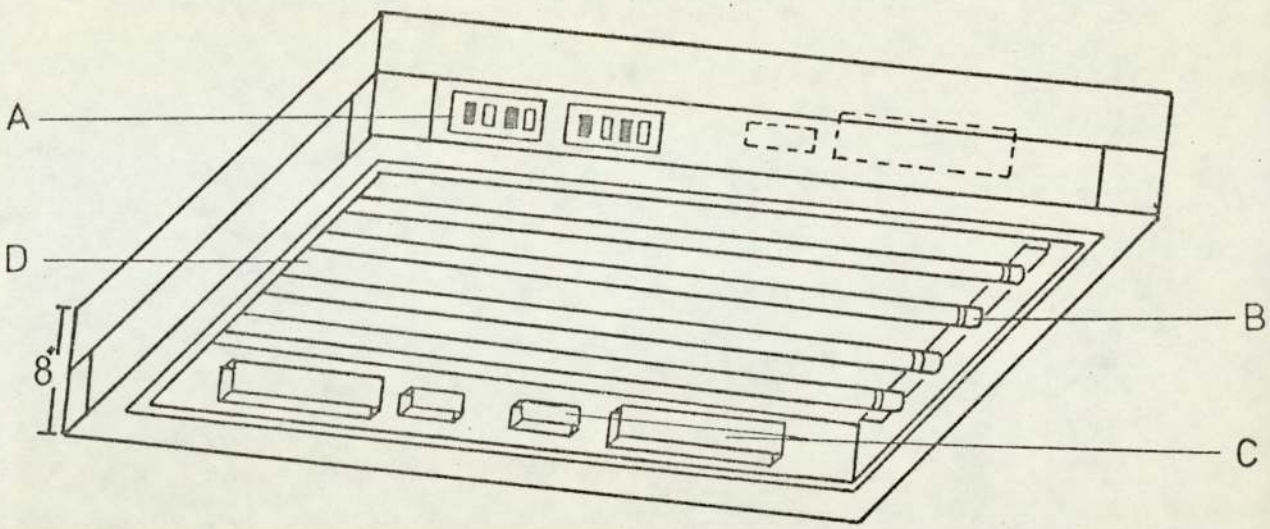
CONSTRUCTION & OPERATION DETAILS

- Overleaf

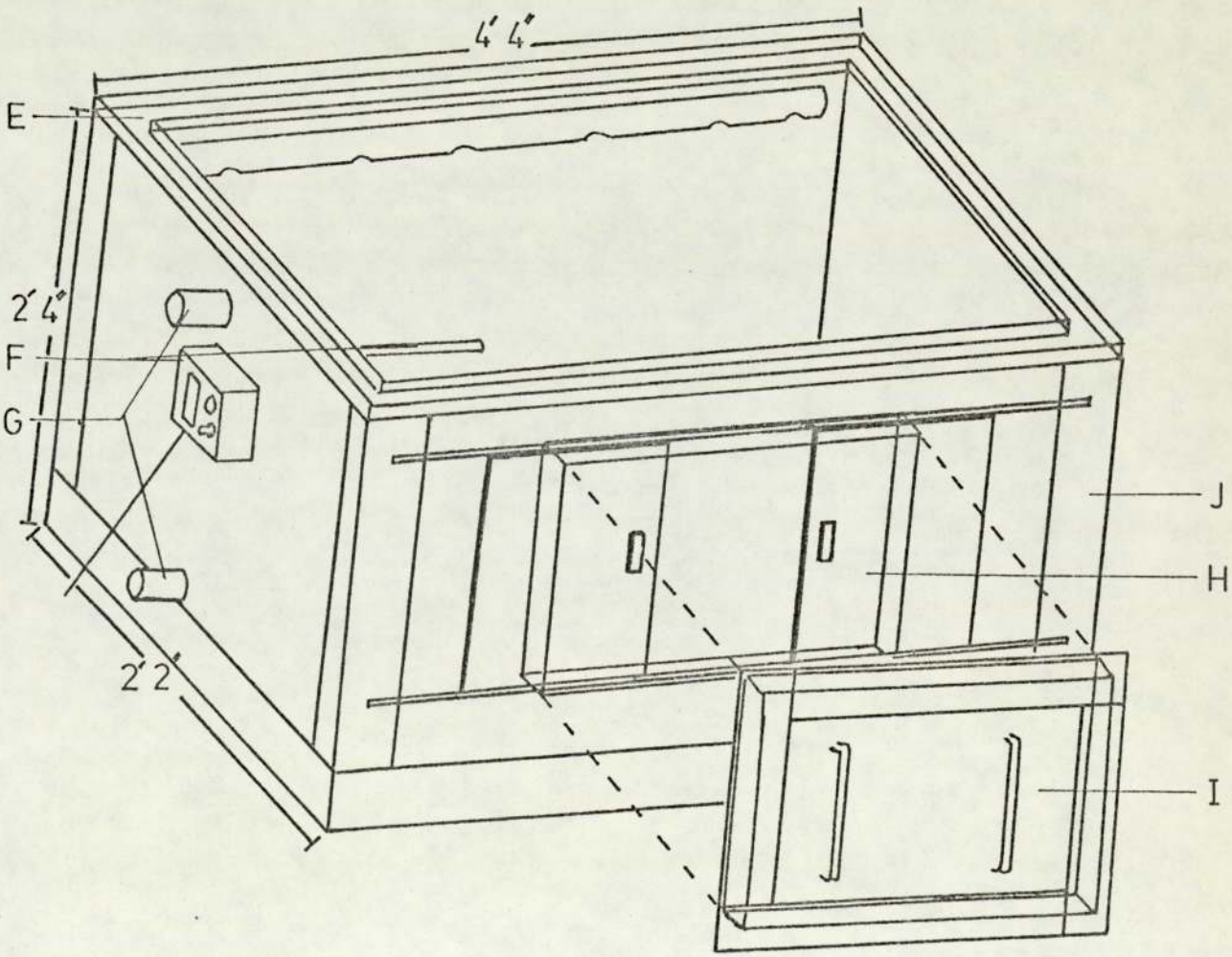
C. MAIN CABINET. INTERNAL FEATURES



A. LIGHTING HOOD



B. MAIN CABINET. EXTERNAL FEATURES



ILLUMINATED CCOLED CABINETS.

Construction and Operation Details.

The cabinets (internal dimensions: 4' x 1'10" x 2'2"; with 4' x 1'10" x 2' headroom) were constructed from a 2" width insulating plastic laminate (Heat Transfer Coefficient 0.044) (D.P.Dawnpress Ltd. Stockport, Cheshire). The top section consisted of a single unit lighting hood (internal dimensions: 4' x 1'10" x 6") which was seated on a foam rubber flange (mounted on the upper rim of the main cabinet) and sealed from the main cabinet by a detachable sheet of clear perspex.

Static incubation within the main cabinet was provided by means of a 'Humex' air-warming system, the control box and temperature sensor rod were mounted on the wall of the cabinet, the air-warming cable passed beneath the bottom wooden support rack and was clipped to its undersurface in a series of longitudinally running loops.

Air circulation was maintained through 2 branched piping systems (c. 2" dia., black plastic pipes perforated by a series of c.$\frac{3}{4}$" dia. holes - pipes from Plastic Constructions Ltd., Birmingham), one below the support rack, the other attached to the wall sections immediately beneath the lighting hood; the twin pipes of both sets passing the length of the cabinets.

The lighting hood contained 4 individually wired 4' 'daylight' fluorescent lights with separate capacitors and ballast linked to externally positioned rocker switches and neons. These formed individual 'instant start' self-starting units (electrical equipment obtained from Walsall Conduits, Aston, Birmingham). The hood size was designed to take a range of cinamoid filters (to investigate possible action spectra for fruit-body formation) clipped or taped to the base

of the perspex sheet . (Coloured filters from Strand Filters, London).

The cabinet was able to accommodate 18 - 6" square by 7" deep white plastic growing containers.

The wall sections consisted of a bonded sandwich of polypropylene sheet (backed with fibreglass reinforcing net - marketed by British Celanese Ltd., London), which provided smooth, clean and chemically resistant internal surfaces and further enabled the wall sections to be sealed internally by the use of polypropylene welding techniques (materials supplied by Plastic Constructions Ltd., Birmingham) forming watertight seams. In fabrication of the insulated wall section by the manufacturers (D.P.Dawnpress Ltd.), the polypropylene/fibreglass sheet is epoxy-resin bonded to expanded polyurethane blocks which are supported by an internal framework of fibreglass uprights; the external surface is finished by a layer of fibreglass / epoxy - resin and coated with a smooth surface white gloss.

Externally the cabinets were supported by a framework of aluminium right-angle (2" x 4") lengths drilled and attached through the outer wall only, by means of specially designed expanding rivets (Linreed Ltd., Birmingham), which formed a 3 pronged anchor attachment at the outer polyurethane/epoxy-resin interface. This arrangement effectively caused minimal interference with the insulating properties of the plastic laminate and produced uninterrupted internal surfaces.

Access was gained by an external 'push-fit' insulated door and an internal sliding clear-perspex observation door forming an internal seal.

Atemperation.

Air over a range of temperatures, (10-50°C) was supplied to the cabinets from a single specially constructed air-conditioning unit. (Lec Refrigeration Ltd.). Provision was made in constructing the

cabinets for recycling air through the blower/conditioner unit in order to attain and maintain low temperatures.

Further Modifications.

The cabinets were prototypes and provided a useful though relatively unsophisticated lighting plus cold temperature facility. However, further modifications required to be introduced to provide (a) improved air distribution; (b) humidity control linked to operation of the conditioner unit (particularly at low temperatures where icing-up occurs); and (c) cooling for the lighting-hood with the maintenance of certain lighting conditions and cabinet temperatures.

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