EFFECTS OF ROOT EXUDATES ON MYCORRHIZAL FUNGI

by

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ABSTRACT

The nutritional requirements of single isolates, obtained from sporophores, of Boletus elegans, Boletus luteus, Boletus bovinus and Boletus variegatus were compared. The results agree closely with those of other workers. The amino acid mixture used in the maximum medium was found to be slightly unbalanced, because the removal of certain acids resulted in improved growth of the fungi. The significance of this with regard to the effects of roots and root exudates is discussed. Boletus elegans was stimulated by low concentrations of calcium, but the responce was small.

Isolates of the four Boletus species were markedly stimulated by excised tomato roots, and in addition Boletus variegatus responded to cell-free root exudates, but not to hot water extracts or homogenates. A quantitative method for the bioassay of exudates, extracts and homogenates was developed.

Chromatography of root exudates revealed a range of amino acids and other compounds. Large amounts of glutamine and asparagine were found, but neither of these compounds stimulated the growth of Boletus variegatus

Boletus variegatus was stimulated slightly by nicotinamide adenine dinucleotide obtained from one source, and also by kinetin and adenine, but in no case was the stimulation great enough to account for the response to roots or root exudates.

Basidiospores of the four Boletus species already mentioned germinated on malt extract agar in the presence of a yeast, Rhodotorula rubra, and also in the presence of tomato, pine and larch roots. Spores of Boletus luteus also germinated in the presence of yeast culture filtrate and excised tomato root exudate.

Boletus elegans interacted markedly with Boletus bovinus and Boletus luteus, and to a lesser extent with Boletus variegatus, when grown on malt extract agar. Similarly Boletus bovinus interacted strongly with Boletus variegatus. The Boletus isolates were inhibited by Marasmius alliaceus and Clitocybe infundibuliformis, both of which are leaf-litter saprophytes, but not by a range of wood-destroying and wood-inhabiting Hymenomycetes. Two other soil saprophytes, a Penicillium species and a Trichoderma species, also inhibited the growth of the Boletus species. Both of these latter antagonists were isolated from soils containing abundent mycorrhizas.

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REVIEW OF THE LITERATURE

The mycorrhizal association.

Ectotrophic mycorrhizas are the results of symbiotic associations between Basidiomycete fungi and forest trees. They appear to be the rule rather than the exception in nature, and are most abundant in acid soils of poor or unbalanced nutrient status.

Many observations made in the field support the view that the symbiotic association benefits the host plant (Melin, 1917; Kessel, 1927; Roeloffs, 1930; Oliveros, 1932; Rayner, 1938, and Dimbleby, 1953). Other observations, made under more easily controlled conditions in nurseries, also support this hypothesis and show that greater amounts of inorganic nutrients are accumulated by infected plants, though only phosphorous has been shown to be greater on an unit dry weight basis (Hatch, 1936 and 1937; Young, 1936; Mitchell, 1937; McComb, 1937 and 1943; Miller, 1938; Rayner *et al.*, 1941; White, 1941; Finn, 1942; Rosendahl, 1942, and Bjorkman, 1944a).

The technique employed by Melin (1936) and Hacskaylo (1953) in which a mycorrhiza between an aseptically grown seedling and a pure culture of a known fungus is synthesized *in vitro*, is now generally accepted as a means of proving whether a fungus forms mycorrhizas. This method

has shown that most mycorrhizal fungi belong to the genera Boletus, Amanita, Tricholoma, Russula, Lactarius and C Cortinarius of the Agaricales. A few are Gasteromycetes such as Scleroderma aurantium and Rhizopogon species, and a few are possibly Ascomycetes. Cenococcum graniforme, probably a sterile Ascomycete, is perhaps the commonest of all mycorrhizal fungi.

The majority of known hosts belong to the three families Pinaceae, Betulaceae and Fagaceae.

The degree of fungal specificity varies widely from the very non-specific Cenococcum graniforme, which is known to associate with 16 host genera, to Boletus elegans, which only occurs with Larix species. A recent review lists all recorded associations including those based on casual observations made in the field (Trappe, 1962).

Studies on the morphology and anatomy of ectotrophic mycorrhizas have been made by a number of workers, most of them in respect of beech (Clowes, 1949, 1950, 1951 and 1954; Harley, 1937 and 1940), and pine (Melin, 1927; Aldrich-Blake, 1930; Hatch et al., 1933; Bjorkman, 1941; Preston, 1943, and Robertson, 1954). Beech mycorrhizas are considered to be the more typical mycorrhizal structures, the root system being racemosely branched with the young non-suberized roots infected. The degree and type of infection for beech

mycorrhizas varies, depending on the soil conditions and the species of fungus involved. Pine differs particularly in that the branching of the root system is dichotomous, a characteristic reaction of the host to the activities of the fungus (Slankis, 1948a and 1950).

Anatomically mycorrhizal roots consist of the host tissues or core, surrounded by fungal tissue or sheath. The latter usually consists of two fairly distinct layers, an inner one of hyphae with intercellular spaces and with cytoplasm and nuclei in evidence, and an outer layer of larger, thick-walled, compacted hyphae. The inner layer is continued as a system of hyphae penetrating the host tissues intercellularly, sometimes as far as, but never beyond, the endodermis. Some intracellular penetration by hyphae does occur as the mycorrhizas age. The outer layer of the sheath is continued as a system of hyphae penetrating into the soil, either extensively or in a more limited manner.

As mentioned earlier, mycorrhizas are particularly abundant in soils of low pH (Hatch et al., 1933), and are less common in base-rich soils (Melin, 1917, and Harley, 1940). From investigations carried out over a number of years Hatch (1937) concluded that the internal nutrient status of the roots was the controlling factor with regard to the intensity of mycorrhizal formation. He found that the

addition of a balanced nutrient mixture to the soil resulted in the production of fewer mycorrhizas.

Bjorkman (1942 and 1944b) considered that light intensity was important. At higher light intensities more soluble carbohydrate was present in the roots of the host trees, and more mycorrhizas were formed under these conditions. Any factors tending to reduce this soluble carbohydrate level, such as increased nutrient supply to the roots, lower light intensity and removal of the phloem by strangulation, resulted in fewer mycorrhizas. The theories of Hatch and Bjorkman seem therefore to be compatible. Other workers have since substantiated these results (Harley, 1948; Robertson, 1954; Harley, et al., 1955, and Wenger, 1955). It has been reported that Cenococcum graniforme forms mycorrhizas at particularly low light intensities (Mikola, 1948).

It is difficult to see, however, how the soluble carbohydrate concentration within a root can induce mycorrhizal formation. Presumably some sort of stimulatory factor would need to be exuded by the roots into the rhizosphere. It has been pointed out by Harley (1959) that light intensity would also influence the production of many substances other than sugars, and that sugars would be, in any case, rather non-specific. It is also worth noting that the soluble carbohydrate content of mycorrhizal roots could be the result of, and not the cause of, mycorrhizal formation

(Handley et al., 1962). Finally the different mycorrhizal associations, some highly specific, others very much less specific, between the fungi and the hosts, must be explained.

The limited information available suggests that most mycorrhizal fungi have little or no ability to exist as saprophytes in the soil. Although Cenococcum graniforme has been reported to be widespread in soils where suitable hosts were absent, this appears to be an exceptional case (Mikola, 1948). A few have been reported to occur as rhizomorphs, e.g. Boletus bovinus, Boletus scaber and Cenococcum graniforme (Rayner et al., 1944, and Levisohn, 1955), and one, Boletus subtomentosus, can fruit in the absence of living host roots (Rommell, 1938 and 1939). It is interesting to note that many species of mycorrhizal fungi are easily cultured in the laboratory, e.g. species of Boletus, Amanita and Tricholoma, while others are not, e.g. Russula species.

Roots of susceptable trees may be infected by mycelium from existing, adjacent mycorrhizas, by basidiospores and possibly by hyphae from resistant structures such as rhizomorphs. The experiments of Robertson (1954), in which seedlings of Pinus sylvestris were inoculated, under controlled conditions, with spores of Boletus granulatus, suggest that basidiospores can be an effective source of inoculum. It has been shown for Pinus sylvestris that established mycorrhizas develop with the growing roots, and can perennate over 2 to 3

years (Robertson, 1954).

The problem of salt uptake by plants infected with mycorrhizas has been studied in some detail. Using a modified version of the technique already described above (see page 6) for synthesizing *in vitro*, it has been shown that radioactively-labelled ions of calcium, sodium, phosphate, ammonium and glutamate can pass from the substratum, *via* living fungal hyphae, to the host tissues (Melin *et al.*, 1950, 1952, 1953a, 1953b, 1955a and 1958a).

The mechanism of phosphate uptake has been studied in excised beech mycorrizas collected from woodland soils (Harley *et al.*, 1950, 1952a, 1952b, 1953, 1954a, 1955, 1958 and 1963). Infected roots absorbed very much more phosphate from solutions than uninfected ones, and did so by an active mechanism which was temperature and oxygen dependent. 90 *per cent.* of the phosphate taken up was found in the fungal sheath but this quantity was reduced either by placing the roots in solutions of high phosphate concentration for absorption, or in phosphate-free solutions after absorption. Low oxygen tensions also had a similar effect, and, with intact seedlings, so did high transpiration rates (Melin, 1958b). Removal of the sheath or slicing the mycorrhizas resulted in increased phosphate uptake by the core.

Movement of phosphate from the sheath to the core, in phosphate-free solutions, was also found to be temperature

and oxygen dependent. It seems that nearly all of the phosphate in the sheath is in an inorganic form, stored at special sites, and there is probably only a very small pool of inorganic phosphate. Any phosphate passing from the sheath to the core goes via this inorganic pool, because only inorganic phosphate enters the core tissues.

Phosphate reaching the core from solutions of low phosphate concentration probably passes directly from the external solution, via the inorganic pool of the sheath, the organic pool remaining undiminished.

In solutions of high phosphate concentration phosphate passes directly to the core via the intercellular spaces of the sheath. This results in increased phosphate uptake by the core. Because high concentrations of phosphate are unlikely to occur generally in soils, this pathway is probably of no importance under natural conditions.

A study of the process of phosphate uptake by intact plants over long periods has confirmed the above hypothesis (Morrison, 1962a and 1962b). Infected plants were found to absorb phosphate at a steady rate, which was independent of their internal phosphate concentrations. In uninfected plants no such independent absorption was found. Absorption of sulphate in both infected and uninfected plants was found to be dependent on internal sulphate concentrations. Infected plants eventually accumulated more phosphate than

uninfected ones.

The fungi.

The fungi of ectotrophic mycorrhizas are considered by Garrett (1950) to be highly specialized parasites evolved to a stage where the symbiotic balance between fungus and host has become obligatory to the fungus *in vivo*. The results of nutritional experiments indicate that, in the soil, the fungi are likely to be restricted to such situations as the rhizosphere. The apparent limited distribution of mycorrhizal fungi as free-living organisms in the soil has already been referred to, though observations of mycelia growing on glass slides in moist soils leave some room for doubt (Kljusnik, 1952).

Some groups of Hymenomycetes are well represented in the literature on nutrition, notably *Boletus* (Melin, 1925, and How, 1948), *Tricholoma* (Norkrans, 1950), *Marasmius* (Lindeberg, 1944), *Mycena* (N. Fries, 1949), *Coprinus* (L. Fries, 1955 and 1956) and *Psalliota bispora* (Treschow, 1944). So also is *Cenococcum graniforme* (Mikola, 1948, and Keller, 1950 and 1952).

Mycorrhizal and non-mycorrhizal Hymenomycetes differ particularly in one aspect of their nutrition, that is in their ability to decompose cellulose and lignin of leaf-litter. Mycorrhizal Hymenomycetes are unable to

decompose these substrates, an exception being Tricholoma fumosum. Non-mycorrhizal Hymenomycetes, on the other hand, including such soil-inhabiting genera as Marasmius, Clavaria, Collybia, Clitocybe and Mycena (Lindeberg, 1944, 1946 and 1949), and also a number of wood-destroying species (Campbell, 1930, 1931 and 1932, and Scheffer, 1936), generally posses this ability.

Mycorrhizal Hymenomycetes are also,unable to produce extracellular polyphenol oxidases, a property which seems to be closely correlated with the ability to decompose lignin (Lindeberg, 1948). Exceptions are Lactarius deliciosus, Boletus scaber and Boletus subtomentosus. Concentrated cold water extracts of leaf-litter are inhibitory towards mycorrhizal Hymenomycetes (Melin, 1946), possibly because of the presence of phenolic compounds (Marley, 1959).

These facts explain in part the limited distribution of mycorrhizal Hymenomycetes in the soil.

The host plant is probably the main source of carbon for mycorrhizal fungi in vivo. The transfer of carbon from host to fungus has been demonstrated in vitro using radioactively labelled carbon dioxide (Melin et al., 1957). Photosynthetic products were found to pass into the fungal sheath. However MacDougal et al. (1944 and 1946) reported that pseudomycorrhizas isolated from their parent trees in the soil survived for many years. More recently it has been shown

that excised beech mycorrhizas can assimilate radioactively-labelled bicarbonate into organic intermediates (Harley, 1964), important perhaps, in view of the fact carbon dioxide levels are sometimes very high in soils (Brierley, 1955).

Hymenomycetes, including mycorrhizal species, are generally similar in their nitrogen requirements, preferring ammonium or organic forms of nitrogen to nitrate nitrogen. Asparagine is particularly well utilized, and also, to a lesser extent, so are urea, peptone, nucleic acids and casein hydrolysate.

Mycorrhizal Hymenomycetes appear to have no special heavy metal requirements. Iron, as might be expected, is required by all Hymenomycetes although the evidence for this is not particularly well documented. Calcium requirements have been demonstrated for species of Tricholoma, Marasmius and Coprinus, although the observed effects could be, in some cases, the result of a protective influence of calcium ions at pHs lower than optimal for fungal growth (Lindeberg, 1944). Requirements for zinc and manganese have rarely been observed, except for zinc in the case of Marasmius scorodonius (Lindeberg, 1944).

Thiamin heterotrophy seems to be the rule among Hymenomycetes. It has been found for species of Amanita, Boletus, Paxillus, Cortinarius and Tricholoma (Melin, 1954),

and for many soil-inhabiting and wood-destroying species (Robbins et al., 1942). A few require the intact thiamin molecule for growth, e.g. Cortinarius glaucopus, but most are able to utilize the separate moieties, thiazole and pyrimidine. Tricholoma species only need the pyrimidine moiety, whereas a few are able to synthesize the thiamin molecule completely, e.g. Clitocybe geotropa, and Hypholoma fasciculare. Different degrees of heterotrophy have been found with different strains of Boletus granulatus (Melin et al., 1941).

Rather few requirements for other vitamins have been found among Hymenomycetes. A partial requirement for biotin has been noted for Rhizopogon roseolus and Cenococcum graniforme among mycorrhizal species, and Marasmius androsaceus and Coprinus narcoticus among non-mycorrhizal species (Melin et al., 1940; Lindeberg, 1941 and 1944, and Nikola, 1948). Collybia dryophila is completely heterotrophic for biotin (Lindeberg, 1946). Partial requirements have also been demonstrated for pantothenic acid, nicotinic acid and para-aminobenzoic acid in Tricholoma species, for nicotinic acid in Lactarius deliciosus (Melin, unpublished), for pantothenic acid in Polyporus texanus (Yusef, 1953) and for inositol in Rhizopogon roseolus (Melin et al., 1939).

Mycorrhizal Hymenomycetes generally respond to amino acid additions, but the limited amount of evidence available suggests that non-mycorrhizal Hymenomycetes behave

similarly. For some species an amino acid mixture is superior to ammonium salts as a nitrogen source, e.g. Cenococcum graniforme (Melin and Nikola, 1948) and Lactarius deliciosus (Melin and Norkrans, 1948). The response of species of Amanita, Boletus, Cortinarius, Lactarius and Rhizopogon to amino acid mixtures appears to be due, particularly, to glutamic acid (Melin, unpublished). Both mycorrhizal and non-mycorrhizal Tricholoma species respond to glutamic acid, some also showing partial requirements for aspartic acid, proline, hydroxyproline, histidine and tryptophane. Partial requirements for tyrosine and phenylalanine in Mycena rubromarginata (N. Fries, 1949) and for tryptophane in Lentinus omphalodes (N. Fries, 1950) have been reported. The balance of the amino acid mixture also seems to be important, some amino acids, notably the aromatic ones, being inhibitory if added singly to the medium, but not if present in a balanced mixture. The partial requirement for glutamic acid can be alleviated by a-ketoglutaric acid (Norkrans, 1953).

Mycorrhizal Hymenomycetes are unaffected by additions of nucleic acid constituents (Melin, 1959), though some non-mycorrhizal species belonging to the genera Polyporus and Mycena respond to a concentration of 50 parts per million adenine (N. Fries, 1951). However Melin (1959) found that adenine and closely related compounds including hypoxanthine, at very low levels, nillified the growth response of a strain

of Boletus variegatus to roots and root exudates.

The effects of roots and root exudates.

Investigations have shown that the growth of the mycelium of a number of mycorrhizal fungi is significantly increased by roots of Pinus sylvestris and a range of non-host plants, notably tomato (Melin, 1954 and Melin et al., 1954). These results were obtained in a medium containing a wide range of amino acids and B-vitamins. Some fungi such as species of Amanita, Boletus, Lactarius and Rhizopogon, only partially require this stimulatory factor from roots, the M-factor of Melin. Others such as Russula species, Cortinarius glaucopus, Pholiota caperata and Lactarius helvus show a complete or nearly complete requirement. The only evidence which shows that non-mycorrhizal Hymenomycetes are not similarly stimulated is experiments in which fungi were cultured in nutrient solutions with Pinus sylvestris seedlings (Lundeberg, 1960). Three Boletus species grew attatched to the roots, and formed pseudomycorrhizal structures with them. Psalliota arvensis on the other hand was not affected, either quantitatively or qualitatively by the roots.

Exudates of excised tomato roots, collected in distilled water over a period of 6 days at 4°C., also stimulated the growth of Boletus variegatus (Melin, 1962 and 1963), and similar results were obtained with excised Pinus

sylvestris roots. Exudates from both types of root had their maximum effect at 5 to 10 units of exudate per 20 ml. of medium. One unit of exudate is that collected from one mg. dry weight of root for 6 days at 4°C. Exudates of Pinus sylvestris roots of seedlings had their maximum effect at less than 5 units per 20 ml. of medium.

Two components seem to be concerned in the stimulatory effect; one, the diffusible factor present in exudates, and the other a non-diffusible factor, which is not extracted from roots by boiling water.

Further experiments with roots and root exudates suggest, also, the presence of an inhibitory component. At exudate concentrations greater than 20 units per 20 ml. of medium (10 units for pine seedling root exudates) this inhibitory component outweighs the effect of the stimulatory components. Exudate collected from damaged roots shows reduced stimulatory activity at all concentrations, suggesting that the inhibitory component diffuses more easily from damaged cells than from intact ones. Autoclaving damaged roots before exudation restores the stimulatory activity to nearly it's original level, possibly, as Melin suggests, by inactivation of a heat-labile factor, which restricts the activity of the diffusible stimulatory component (Melin, 1963).

Efforts to identify the diffusible stimulatory factor have so far been unsuccessful. Among a number of

sugar phosphates fructose, 1-6, diphosphate stimulates the growth of Boletus variegatus and the non-mycorrhizal Collybia velutipes, but it is not known whether it could replace the root effect (Nilsson, 1956). More recently, in a report to the Swedish Council of Natural Science, it was stated that nicotinamide adenine dinucleotide replaces the diffusible stimulatory factor (Nilsson, 1960).

Other unidentified growth factors.

Ash and dilute cold water extracts of humus stimulate the growth of mycorrhizal fungi (Melin, 1946), the former, it is suggested, by the presence of calcium, and the latter by some organic component. As has already been mentioned high concentrations of water extracts are inhibitory.

Extracts of Calluna peat are also inhibitory towards mycorrhizal fungi, an effect which is nullified by the presence of soil saprophytic microorganisms. Such organisms probably destroy or remove the toxic substances involved. Boletus scaber, which is a polyphenol oxidase producing mycorrhizal fungus, commonly associates with birch on Calluna heathlands, and is relatively immune to Calluna extracts (Dimbleby, 1953).

The soil microflora and mycorrhizal fungi.

It is known that certain Pseudomonas species found

in soils are essential for the *in vitro* synthesis of endotrophic mycorrhizas (Mosse 1962). As might be expected a number of observations point to the possible importance of soil microorganisms in the formation of ectotrophic mycorrhizas.

The mycorrhizal fungi may be directly influenced by antibiotics produced by actinomycetes (Muller, 1960) and fungi (Rayner *et al.*, 1944, and Levisohn, 1957). Muller found that actinomycetes and antibiotics such as actinomycin, chloromycetin, streptomycin, penicillin and patulin, generally had greater effects on proven mycorrhizal fungi, e.g *Boletus bovinus*, *Boletus luteus* and *Boletus variegatus*, than on doubtful mycorrhizal formers, e.g. *Gomphidius* species. Rayner *et al.* found that in soils on Wareham Heath rhizomorphs of *Boletus bovinus* did not infect available hosts. This was later ascribed to the production of gliotoxin by *Penicillium jenseni*, a common fungus in these soils (Brian *et al.*, 1945). Levisohn (1957) observed that in mixed cultures, *Boletus* species were inhibited, to a greater or lesser extent, by *Alternaria tenuis*. Pseudomycorrhizal fungi such as *Mycelium radicis atrovirens* and a *Rhizoctonia* species were not inhibited. Similar results were obtained with culture filtrates of *Alternaria tenuis*.

Mycorrhizal fungi may also be influenced more indirectly by the activities of soil saprophytes. The inactivation of inhibitory substances in *Calluna* extracts has

already been mentioned (see page 20).

The role of microorganisms in basidiospore germination will be dealt with in a later section.

There is some evidence that the rhizosphere microfloras of mycorrhizal and non-mycorrhizal roots differ (Katznelson et al., 1962). It was found that more bacteria were present in the rhizospheres of mycorrhizal roots of Yellow Birch seedlings, compared with uninfected roots, and that a greater proportion of these bacteria were slower growing and more exacting in their nutritional requirements. The fungal population of the rhizospheres of mycorrhizal roots was also qualitatively different from that of uninfected roots, species of Pythium and Fusarium being absent from the former.

Finally there is some evidence that mycorrhizas may protect host roots against infection from wood-destroying parasitic Hymenomycetes (Rypacek, 1960). In culture Boletus variegatus was found to be antagonistic towards some parasitic wood-destroying species, e.g. Paxillus atromentosus, Fomes marginatus and Phellinus hartigi, but not towards some wood-decomposing saprophytic fungi, e.g. Osmoporus odoratus, Schizophyllum commune and Xylaria hypoxylon.

Basidiospore germination.

The rather limited amount of information on the germination of basidiospores of mycorrhizal fungi suggests

that these spores do not germinate easily, and will only do so in the presence of an activator, produced by certain living microorganisms. The spores of a number of species of Lycoperdon, Scleroderma, Boletus, Tricholoma, Amanita, Paxillus, Hydnum and Craterellus, many of which are proven mycorrhizal fungi, germinate only in the presence of certain yeasts, namely Torula Suganii, Torula sanguinea and an unnamed species (N. Fries, 1941 and 1943).

Melin (1955 and 1962) observed that roots of tomato and Pinus sylvestris also stimulate the germination of these spores. Spores of species of Russula, Lactarius and Cortinarius and also Amanita muscaria and Paxillus involutus, will only germinate in the presence of roots, and not in the presence of yeasts. In some cases the requirement is even more specific, e.g. spores of Russula adusta and Russula rosacea will only germinate with Pinus sylvestris roots. Spores of some species fail to germinate at all, again notably those of certain Russula species. At the other end of the scale spores of some Tricholoma, Amanita and Boletus species and also Paxillus prunulus are able to germinate in the absence of an activator (N. Fries, 1943; Khudiakov, et al., 1951, and Melin, 1962).

Some fungi are able to stimulate basidiospore germination. These include Cladosporium species, Trichosporium heteromorphum, Cenococcum graniforme and Mycelium radicis

atrovirens. Sporophore extracts of various Basidiomycetes also stimulate the spores of Boletus luteus and Boletus variegatus to germinate.

Germination is rarely above 0·1 per cent. for the spores of any of the species mentioned, and the time for germination is usually many days.

Nothing is known of the nature of the activator which stimulates spore germination. N. Fries (1941) suggested that, in the case of Lycoperdon umbrinum, the activator functions in two ways; one, by nullifying certain inhibitory substances in the malt extract agar, and two, by directly stimulating the spores. Melin has suggested that the M-factor, which stimulates mycelial growth, and the germination activator may be identical.

INTRODUCTION

Four closely related species of Boletus were chosen for these investigations, Boletus elegans Schum ex Fr., Boletus luteus L. ex Fr., Boletus bovinus L. ex Fr. and Boletus variegatus Sow. ex Fr. These species are easily cultured, and a certain amount is already known about their physiology in vitro. Further their symbiosis with specific tree species is well established.

The purpose of the investigations is three-fold. First, to examine in some detail nutrition in vitro, because data on this subject, for Boletus species, is rather fragmentary.

Secondly, to repeat the experiments and extend the studies of Melin on the M-factor from roots, which stimulates the mycelial growth of mycorrhizal fungi in vitro.

Thirdly, to repeat and extend the studies of N. Fries and Melin on the activator which stimulates basidiospore germination of many Basidiomycetes, including both mycorrhizal and non-mycorrhizal species.

MATERIALS AND METHODS

The fungi.

Young sporophores of Boletus elegans, Boletus luteus, Boletus bovinus and Boletus variegatus were collected from the places and on the dates shown in Table 1. Pure cultures were established and kept at 25°C. on malt extract agar. Tissue of the pileus taken from just above the stipe was found to be particularly suitable for isolation.

The malt extract agar contained malt extract (Oxoid) - 15g., agar (Davis, type P) - 15g. and distilled water to 1 litre. The following two media were also used: Lindeberg agar (Lindeberg, 1948), which contained glucose - 10g., NH_4Cl - 0·5g., KH_2PO_4 - 0·5g., $MgSO_4.7H_2O$ - 0·5g., malt extract (Oxoid) - 2·5g., agar (Davis type P) - 15g. and distilled water to 1 litre; and modified Lindeberg agar, which contained glucose - 5g., NH_4Cl - 0·5g., KH_2PO_4 - 0·5g., $MgSO_4.7H_2O$ - 0·5g., malt extract (Oxoid) - 5g., agar (Davis, type P) - 15g. and distilled water to 1 litre.

Analar reagents were used where these were available. The media were autoclaved at 15 p.s.i. for 15 minutes.

Malt extract agar and Lindeberg agar were used during the first few months, but later stock cultures were maintained on the modified Lindeberg agar. Isolates of

TABLE 1. The Boletus isolates.

Species	Isolate No.	Locality and date collected
B. elegans	1	nr Silwood Park, 10/61
" "	52	Black Nest, 8/62
" "	63	Silwood Park, 8/62
" "	67	nr Birmingham, 9/62
" "	77	Swinley, 10/62
" "	81	Bedgebury, 10/62
B. bovinus	42	Bedgebury, 11/61
" "	70	Silwood Park, 10/62
" "	73	Swinley, 10/62
" "	78	Windsor Park, 10/62
B. luteus	19	Silwood Park, 10/61
" "	44	Bedgebury, 11/61
" "	74	Swinley, 10/62
" "	79	Windsor Park, 10/62
" "	83	Bedgebury, 10/62
B. variegatus	50	Bedgebury, 11/61
" "	55	Bedgebury, 8/62
" "	72	Swinley, 10/62
" "	82	Bedgebury, 10/62

Boletus elegans were subcultured every 4 weeks, Boletus luteus and Boletus variegatus every 3 weeks and Boletus bovinus every 2 weeks. Stock cultures of certain isolates, namely Boletus elegans (1), Boletus luteus (19), Boletus bovinus (42) and Boletus variegatus (50), used in the nutritional experiments, were also kept on malt extract agar under mineral oil (Liquid Paraffin, S.G. 0·830-0·870). These isolates will subsequently be referred to as the primary isolates.

The mycelial morphology of all isolates, grown on malt extract agar, was examined, both macroscopically and microscopically. Details are given in Appendix A, and the general appearance of colonies can be seen in Fig. 1. The primary isolates were also examined on basic and maximum ionagar (see page 41 for details of these media), and the general appearance of the colonies can be seen in Fig. 2. Again details are given in Appendix A.

Biochemical tests were also carried out on all isolates, grown on malt extract agar, and aged about 3 weeks. Details are given in Appendix B.

The synthesis of mycorrhizas in vitro.

The technique first used by Melin (1921) and later modified by Macskaylo (1953) was adopted, with further slight modifications.

Seeds of Pinus sylvestris and Larix decidua were

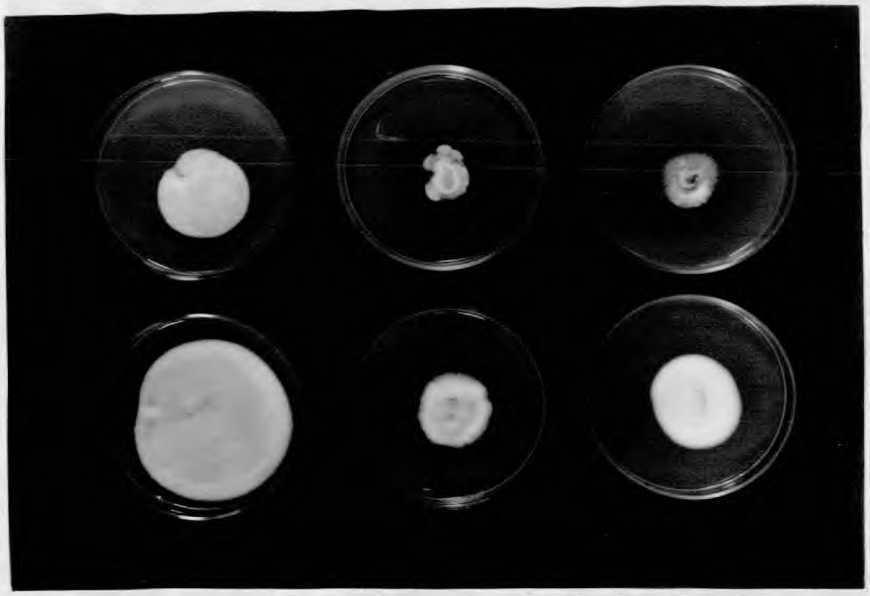


Fig. 1. Boletus elegans isolates aged 3 weeks on malt extract agar: 1 (top left), 52 (top centre), 63 (top right), 67 (bottom left), 77 (bottom centre) and 81 (bottom right).

Fig. 1. Boletus luteus isolates aged 3 weeks on malt extract agar: 19 (top left), 44 (top centre), 74 (top right), 79 (bottom left) and 81 (bottom right).

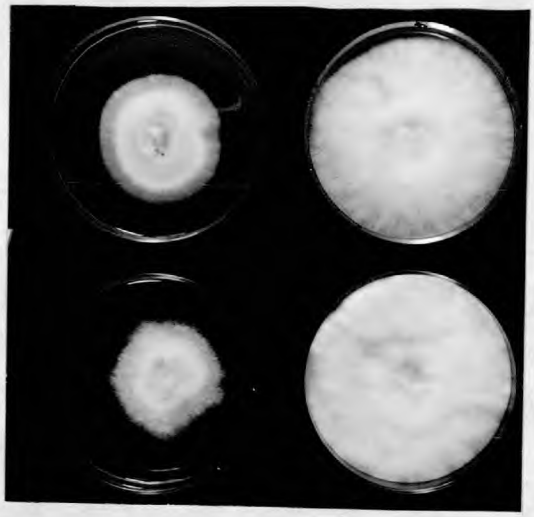


Fig. 1. Boletus bovinus isolates aged 2 weeks on malt extract agar: 42 (top left), 70 (top right), 73 (bottom left) and 78 (bottom right).

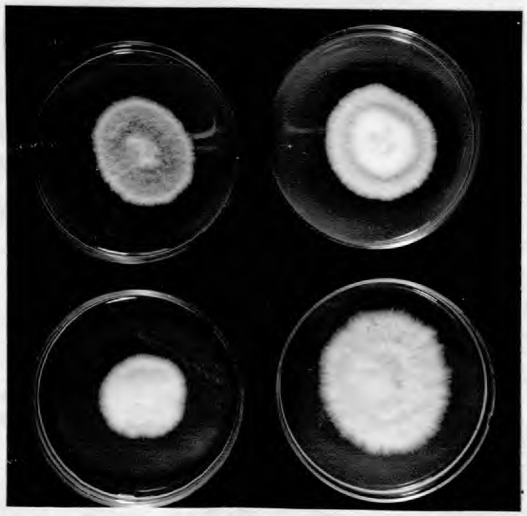


Fig. 1. Boletus variegatus isolates 3 weeks on malt extract agar: 50 (top left), 55 (top right), 72 (bottom left) and 82 (bottom right).

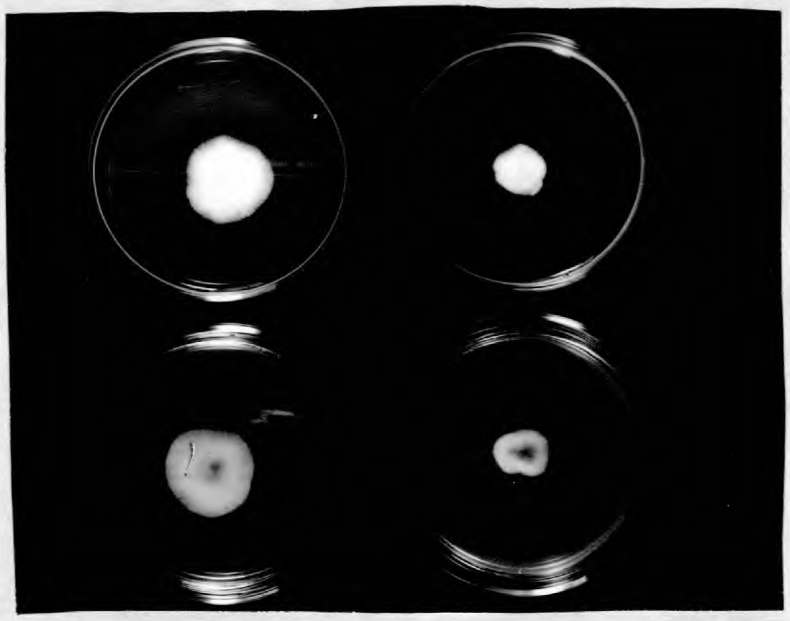


Fig. 2. Boletus elegans (1) on basic ionagar seen from above (top left) and below (bottom left) and on maximum ionagar seen from above (top right) and below (bottom right).

Fig. 2. Boletus luteus (19) on basic ionagar seen from above (top left) and below (bottom left) and on maximum ionagar seen from above (top right) and below (bottom right).

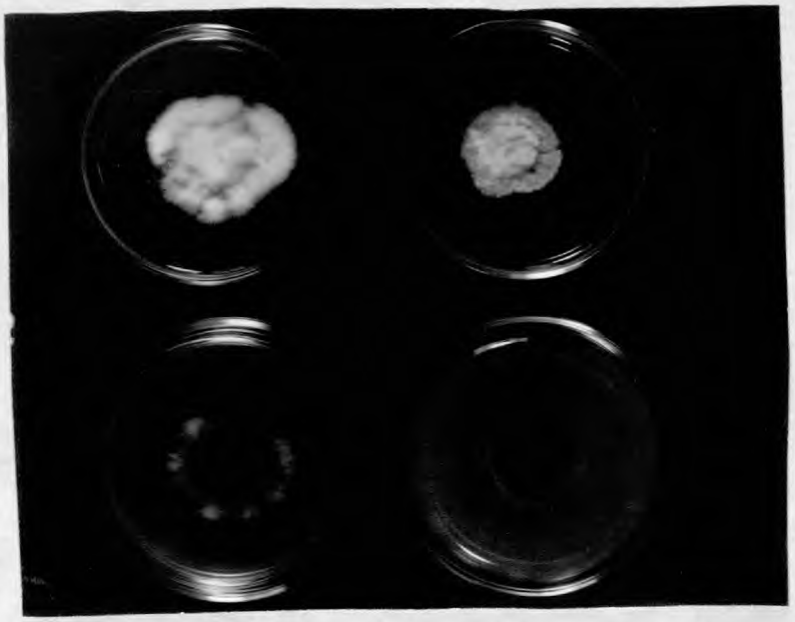


Fig. 2. Boletus bovinus (42) on basic ionagar seen from above (top left) and below (bottom left) and on maximum ionagar seen from above (top right) and below (bottom right).

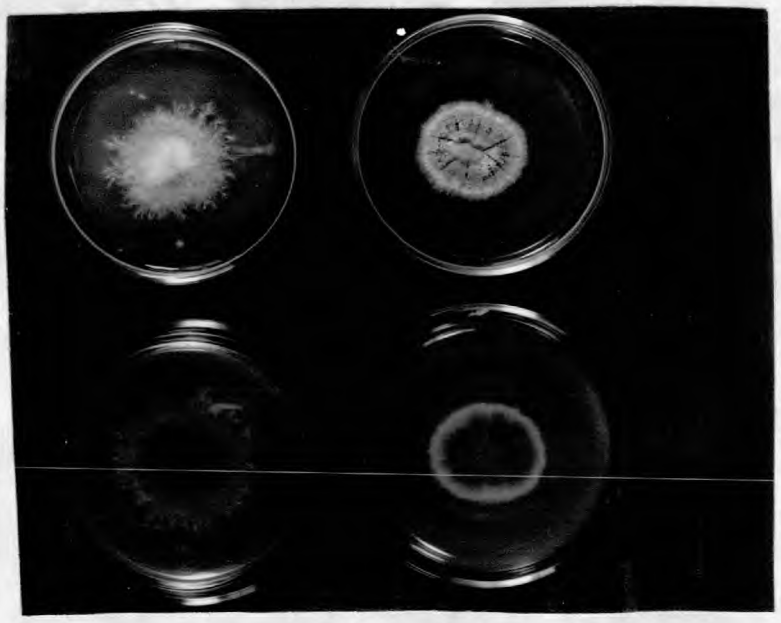


Fig. 2. Boletus variegatus (50) on basic ionagar seen from above (top left) and below (bottom left) and on maximum ionagar seen from above (top right) and below (bottom right).

surface sterilized, and were then germinated as described below (see pages 36 and 52). Two seedlings, aged about 5 days, were transferred to a 500ml. conical flask containing 15g. of vermiculite (medium grade), and 120ml. of a nutrient solution containing glucose - 2·5g., $(NH_4)_2HPO_4$ - 0·25g., KH_2PO_4 - 0·5g., $CaCl_2$ - 0·05g., NaCl - 0·025g., $MgSO_4.7H_2O$ - 0·15g., ethylamine-diaminetetra-acetic acid (Fe salt) - 6mg., thiamin HCl - 0·015mg. (added with fungus) and distilled water to one litre.

In some experiments, following the method of Macskaylo, the pH after autoclaving was high, about 7·2. In other experiments the addition of 10ml. of normal hydrochloric acid to each flask gave a final pH of 5·2.

Sieved and non-sieved mycelial suspensions, at 10ml. per flask, were used in some experiments, whereas agar inocula were used in others. Details of the preparation of these ~~media~~ inocula are given below (see page 43).

In some experiments, again following Macskaylo's method, flasks were inoculated with the fungus when the seedlings were 8 to 12 weeks old. In others the flasks were inoculated when the seedlings were one week old. In the former case seedlings were removed for examination when they were about 18 weeks old, and in the latter case when they were about 12 weeks old.

The seedlings were freed from vermiculite and washed in tap water, care being taken to avoid damaging or

breaking-off the lateral roots. Infected roots were then transferred to fixative containing formalin - 5ml., glacial acetic acid - 5ml. and 70 per cent. alcohol - 90ml.

The fixative was removed by washing twice in 63 per cent. ethanol, and the material was then dehydrated by passing through a series of solutions containing varying proportions of water, methylated spirits, tertiary butyl alcohol and ethanol. The material was finally transferred from pure tertiary butyl alcohol to a mixture of equal parts of tertiary butyl alcohol and paraffin oil. Paraffin wax (melting point 56°C.) was used for embedding.

Sections, 10 to 12 microns thick, were cut and mounted on clean glass slides with Haupts adhesive. They were then rehydrated, after removal of the wax with xylol, by passing through a series of solutions containing varying proportions of ethanol and water, and finally into pure water.

Various staining techniques were tried including cotton blue in lactophenol, picroaniline blue and safranin (aqueous and in methyl-cellosolve), fast green and safranin (aqueous and in methyl-cellosolve) and orsellin BB and crystal violet. The best results were obtained, however, with carbol-thionin and orange G.

Sections were placed in carbol-thionin for at least one hour, and were then transferred to absolute alcohol, via

a series of alcohols. The differentiation time in orange G was fairly critical, 30 seconds giving the best results. The slides were finally rinsed in absolute alcohol and the sections mounted in euparol.

The culture of excised tomato roots.

Undamaged tomato seeds, variety Ailsa Craig, were selected, and were immersed for 2 minutes in 0·02 per cent. mercuric chloride containing a trace of Tween 80. They were then washed in 6 changes of sterile distilled water for 10 minutes each change, and were placed on dilute malt extract agar (containing 0·5 per cent. malt extract). The seeds germinated in 3 days.

After one week radical tips, one cm. long, were excised with a sharp scalpel, and were transferred to 250ml. conical flasks containing 50ml. modified White's medium, which consists of sucrose - 20g., $Ca(NO_3)_2.4H_2O$ - 0·2g., $MgSO_4.7H_2O$ - 0·36g., KNO_3 - 0·08g., KCl - 0.065g., Na_2SO_4 - 0·2g., $NaH_2PO_4.2H_2O$ - 0·0165g., KI - 0·75mg., $FeSO_4.7H_2O$ - 2·5mg., $MnSO_4.4H_2O$ - 0·5mg., $ZnSO_4.7H_2O$ - 1·5mg., H_3BO_3 - 1·5mg., thiamin HCl - 0·1mg., nicotinic acid - 0·5mg., pyridoxin HCl - 0·1mg. and distilled water to 1 litre.

Analar reagents were used where available. The medium, which differed from White's medium proper in the ommision of glycine, was sterilized by autoclaving at 15 p.s.i.

for 15 minutes.

The roots were subcultured every week, the inocula being sectors consisting of lateral roots, bearing their 5 to 8 youngest secondary laterals, with the primary lateral meristem removed (Fig. 3).

Three clones of roots were used during the course of the investigations, because each tended to lose vigour fairly rapidly after about 9 to 12 months. Some characteristics of the clones are given below.

Clone.	Life span.	Dry weight in 7 days.
C	13 months	7•5mg.
X	8 months	4•5mg.
N	12 months	6•6mg.

The culture of excised Pinus sylvestris roots.

Pinus sylvestris seeds were obtained from Thompson and Morgan Ltd of Ipswich. Only dark, non-mottled seeds were selected for use, because the others are believed to germinate poorly (Slankis, 1948). Undamaged seeds were immersed in 0•1 per cent. mercuric chloride containing a trace of Tween 80 for 5 minutes. They were washed in 6 changes of sterile distilled water, each of 10 minutes duration, and placed on 0•5 per cent. malt extract agar. About 60 per

Fig. 3. 7 day old excised tomato root (Clone C) and sector.

cent. of the seeds germinated in 7 days.

After 10 days radical tips, one cm. long, were transferred to 150ml. conical flasks containing 20ml. of Slankis' medium (Robbins et al., 1936), which contained glucose - 30, 40, 50 or 60g., $Ca(NO_3)_2.4H_2O$ - 0·05g., $MgSO_4.7H_2O$ - 0·01g., $KH_2PO_4.2H_2O$ - 0·0165g., $FeSO_4.7H_2O$ - 1mg., $MnCl_2$ - 0·1mg., $ZnCl_2$ - 0·1mg., H_3BO_3 - 0·1mg., thiamin HCl - 0·05mg., biotin - 0·05mg. and distilled water to 1 litre.

Analar reagents were used where available, and the medium was sterilized by autoclaving at 15 p.s.i. for 15 minutes.

The roots grew very slowly at all glucose concentrations, giving a six-fold increase in root length after 30 weeks, about 2·6mg. dry weight, a figure which compares well with Slankis' results (Slankis, 1948). This slow growth rate meant that Pinus sylvestris roots could be used only in a few experiments.

10^{-5} molar indolyl-acetic acid was sometimes added to the mediumin an attempt to stimulate root growth, because Ulrich (1962) found that 2 weeks pretreatment with the hormone subsequently improved the growth of excised Pinus ponderosa roots. However, no stimulation of Pinus sylvestris roots was obtained.

The culture of tomato seedlings.

Seeds were selected, sterilized and placed on 0·5 per cent. malt extract agar as described above (see page 35). As soon as the seeds had germinated they were transferred to growth tubes, each of which consists of a boiling tube containing a close-fitting glass cylinder with a piece of nylon net fastened over the top, and with a cotton-wool plug. A growth tube is shown in Fig. 4. The net and the elastic band used to hold the net in place were leached before use by autoclaving and leaving overnight in fresh distilled water.

45 to 50ml. of nutrient medium was placed in each tube so that the liquid level was 2mm. below the net. The medium is based on that used by other workers (Mulder, 1948, and Clark et al., 1932), and consists of $Ca(NO_3)_2.4H_2O$ - 0·2g., $MgSO_4.7H_2O$ - 0·2g., $(NH_4)_2SO_4$ - 0·2g., KH_2PO_4 - 0·5g., ethylenediaminetetra-acetic acid (Fe salt) - 2·5mg., $MnSO_4.4H_2O$ - 0.25mg., $CuSO_4.5H_2O$ - 0·05mg., $ZnSO_4.7H_2O$ - 0·05mg., H_3BO_3 - 0·5mg., $Na_2MoO_4.2H_2O$ - 0·02mg. and distilled water to 1 litre.

Analar salts were used where available, and the medium was sterilized by autoclaving at 15 p.s.i. for 15 minutes.

Three seedlings were grown in each tube, and the tubes were wrapped in black paper to the level of the net.

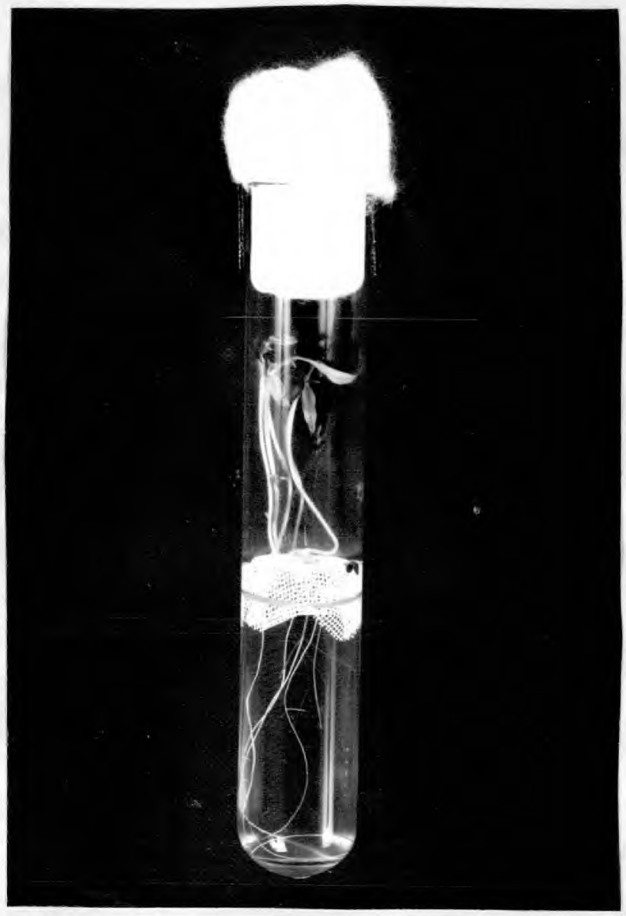


Fig. 4. Growth tube for culture of tomato seedlings.

Experimental media.

The basic medium used in these experiments was that used by Melin (Melin et al., 1954), and consists of glucose - 20g., ammonium tartrate - 0·5g., $MgSO_4.7H_2O$ - 05g., KH_2PO_4 - 1g., $FeSO_4.7H_2O$ - 5mg., $ZnSO_4.7H_2O$ - 5mg., thiamin HCl - 0·05mg. and distilled water to 1 litre.

Analar salts were used where available, and the medium was sterilized by autoclaving at 15 p.s.i. for 15 minutes.

The maximum medium consisted of the basic medium plus an amino acid mixture and a vitamin mixture. The amino acid mixture consists of glycine - 2·5mg., dl-a-alanine - 95 mg., dl-valine - 39·5mg., l-leucine - 24·5mg., dl-isoleucine - 24·5mg., dl-phenylalanine - 19·5mg., l-tyrosine - 33mg., l-tryptophane - 11mg., l-glutamic acid - 161mg., l-aspartic acid - 20·5mg., l-proline - 45·5mg., l-hydroxyproline - 1mg., dl-serine - 2·5mg., dl-threonine - 17·5mg., l-cystine - 1·5mg., dl-methionine - 17mg., l-arginine - 19mg., l-histidine HCl - 12·5mg., l-lysine HCl - 30mg. to 1 litre of medium.

The vitamin mixture consists of riboflavin - 0·05mg., Ca pantothenate - 0·05mg., folic acid - 0·05mg., nicotinic acid - 0·05mg., para-aminobenzoic acid - 0·05mg., pyridoxine HCl - 0·05mg., biotin - 0·0005mg. and choline chloride - 0·5mg. and inositol - 50mg. to 1 litre of medium.

Both the amino acid and the vitamin mixtures were

sterilized by autoclaving at 15 p.s.i. for 15 minutes, and were added to the basic medium aseptically.

The pH of the basic medium is 5.0 and that of the maximum medium 4.6.

Basic ionagar and maximum ionagar were prepared by adding 12g. Oxoid Ionagar No. 2 to a litre of basic or maximum medium.

Unless otherwise stated, 20ml. of liquid medium and 20ml. of ionagar medium were used in 150ml. conical flasks and in 9cm. petri dishes respectively.

The carbon sources used in the investigations are listed below. The pH adjustments were made prior to sterilization, to give a pH after sterilization of 5.0.

Carbon source and origin.	pH adjustment per 400ml.
Glucose (B.D.H.)	nil
Fructose (B.D.H.)	"
Galactose (B.D.H.)	"
Mannose (B.D.H.)	"
Xylose (B.D.H.)	0.3ml. N/1 NaOH
Mannitol (B.D.H.)	nil
Maltose (B.D.H.)	"
Sucrose (B.D.H.)	"
Na acetate (B.D.H.)	10ml. N/1 HCl
Na malate (B.D.H.)	5ml. N/1 HCl

Soluble starch (Hopkins & Williams)	nil
Pectin (Sunkist Growers)	2ml. N/1 NaOH
Na polypectate (Sunkist Growers)	nil
Carboxy-methyl-cellulose (Hercules)	1ml. N/1 HCl
Wood cellulose (Brown Co.)	nil
Esparto hemicellulose	3ml. N/1 HCl

Fructose and xylose were sterilized by micropore filtration and the rest by steaming.

Calcium chloride was autoclaved separately and added aseptically to the maximum medium.

Glutamine, asparagine, nicotinamide adenine dinucleotide, kinetin and adenine were all sterilized by micropore filtration and were added aseptically ot the maximum medium. Nicotinamide adenine dinucleotide and kinetin were made up fresh as required.

Fungal inocula.

The two main types used were mycelial suspensions, which were used for the experiments with roots and root exudates, and agar inocula, which were used in all other nutritional experiments.

Mycelial suspensions were prepared form 10 day old colonies floating on basic medium. 15 colonies were

were transferred to a 150ml. conical flask containing glass beads (6mm. diameter) and 15ml. sterile distilled water. The mycelia were then macerated by hand-shaking for 1½ minutes. Sieved mycelial suspensions were prepared by filtering mycelial suspensions through butter muslin, using the apparatus illustrated in Fig. 5.

Agar inocula were prepared by cutting pieces, 2mm. square, from the periphery of a 10 day old colony on basic ionagar. Leached inocula were prepared by floating agar inocula on sterile distilled water for 12 hours before use. All reducing sugar was removed by this method, as shown by a negative reaction to the modified Somogyi reagent (Nelson, 1944), and probably most other nutrients as well.

Excised roots in experiments.

In experiments with excised tomato roots in basic and maximum medium whole 7 day old roots were used.

For experiments carried out on maximum ionagar sectors of the type normally employed in subculturing were used.

In experiments with killed roots either whole roots or sectors were immersed for 5 seconds in boiling water, and were then transferred to flasks or agar plates.

Excised tomato root extract was prepared by placing 7 day old roots in boiling distilled water for 20 minutes,

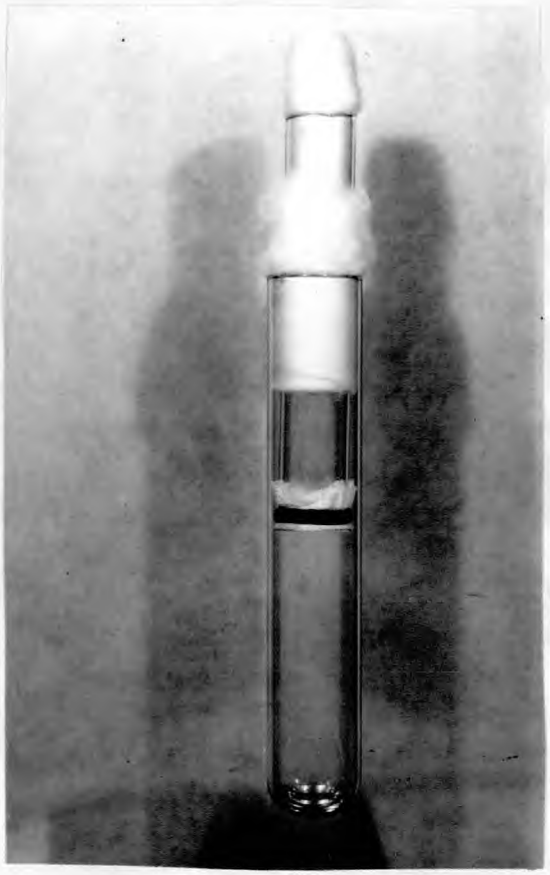


Fig. 5. Apparatus for preparing sieved mycelial suspension.

and then removing the roots from the extract and finally the debris by centrifugation.

Homogenates of excised tomato roots were prepared by macerating 7 day old roots in a Waring blender for 2 minutes, and then filtering off the mush and finally removing the debris by centrifugation. Previously extracted homogenate was prepared by extracting with boiling distilled water for 20 minutes prior to maceration.

Water-soluble ash was prepared by incinerating roots in a porcelain crucible, over a bunsen flame, and dissolving the residue in very dilute hydrochloric acid.

The concentrations of the extract, homogenates and ash were then adjusted to give the equivalent of 10mg. dry weight of root per ml. of solution. The solutions were sterilized by micropore filtration.

Excised tomato root exudates.

7 day old excised tomato roots were placed in sterile distilled water, or in dilute White's medium, at 25°C. for 7 days. The exudate was then concentrated at 40°C. to give the equivalent of 10mg. dry weight of root per ml., and was sterilized by micropore filtration.

Experimental techniques.

Unless otherwise stated the experiments were carried

out at 25°C. in the dark, and the incubation period was 7 days.

Plates of ionagar were poured, and in experiments with root exudates, extract, homegenates and ash a well-plate technique was used. Either glass wells, internal diameter 5mm. and height 5mm., were placed on the agar surface, or wells of similar dimensions were cut out of the agar. 0•1ml. of the solution to be tested was put in each well. Plates were inoculated with either 20 microlitre drops of sieved mycelial suspension placed 15mm. from the edge of the well, or with 0•25ml. of sieved mycelial suspension spread uniformly over the agar surface. In the latter, auxanographic technique, the plates were inoculated one day before preparing and filling the wells, and were dried overnight at 30°C. The auxanographic technique was also used for experiments with root sectors.

Growth assessment.

For all experiments in liquid media mycelia were strained into butter muslin, washed in distilled water and dried at 70°C. for 24 hours on tared aluminium foils. In experiments with roots the fungus was separated as far as possible from the roots. In experiments where polysaccherides were used as carbon sources, careful washing was necessary to free the mycelium from unused polysaccheride.

The diameters of colonies growing on agar media were measured to the nearest mm., and always along the same

line through the centre of the colony, because the colonies were not always perfectly circular.

The pH values of the liquid media were measured at the the end of the experiments. Generally the pH of the maximum medium fell to not less then 4.0.

Means are based on 4 replicates. Standard errors are given with the means except where results are given only in the form of graphs. The standard deviation for each point on a graph is represented by the vertical line through the point. The difference between two means is considered to be significant when the calculated value of t exceeds the value given by the 5 per cent. probability level curve, for the number of degrees of freedom in question.

Chromatography of root exudates.

Excised tomato root exudates were concentrated at 40°C., to give the equivalent of 100mg. dry weight of root per ml.

The techniques used are those based on Smith (1960). All chromatograms were run on Whatman No. 1 "chromatography" paper, ascending, in a Universal Chromatank, except for inorganic ions, which were run, descending, for 40cm. overnight. The following solvents were used.

Chemical group.	Solvent.
Amino acids	n-butanol, acetic acid, water (1st solvent)
	phenol, ammonia (2nd solvent)
Sugars	iso-propanol, water
Phenols	2% acetic acid
Alkaline earth metals	iso-propanol, methanol, formic acid, ammonium formate, water

No time for equilibration was necessary, except in the case of the solvent for the alkaline earth metals, for which 4 hours saturation of the tank prior to running was allowed.

Chromatograms were developed over 20cm., and the solvents were blown off with cool air for about 30 minutes.

The locating reagents used for the amino acids were ninhydrin, isatin and Ehrlich reagent; for sugars aniline-diphenylamine, anisidine reagent, naphthoresorcinol reagent and Elson-Morgan reagent; for phenols ferric chloride-potassium ferricyanide reagent and diazotized sulphanilic reagent, and for alkaline earth metals oxine reagent.

Collection of basidiospores.

Basidiospore collections from single sporophores

of Boletus elegans, Boletus luteus, Boletus bovinus, Boletus variegatus and Boletus scaber were made. Collections made from one compact group of sporophores are given the same reference number, and are listed in Table 2.

TABLE 2. The basidiospore collections.

Species	Collection	Locality and date collected
B. elegans	1, a-d	Bedgebury, 9/63
" "	2, a-h	Swinley, 10/63
" "	3, a-d	High Standing Hill, 10/63
B. bovinus	1, a-c	Bedgebury, 9/63
" "	2, a-b	Swinley, 10/63
B. luteus	1, a-b	Swinley, 10/62
" "	1a	Bedgebury, 9/63
" "	2, a-d	Bedgebury, 9/63
" "	3, a-d	High Standing Hill, 10/63
B. variegatus	1a	Bedgebury, 9/63
B. scaber	1, a-c	Swinley, 10/63

Collections were stored at laboratory temperature.

Germination of basidiospores.

A technique using agar plates, instead of the more

conventional methods of measuring spore germination in liquid drops on slides, was preferred, because the spores always take a long time to germinate, and because the percentage germination rarely exceeds 0·1 per cent. It would be difficult to maintain liquid drops for such a period, and to count the germinated spores. Also spores would tend to aggregate together in liquid drops over a long period of time, and this might have some effect on germination by autostimulation or autoinhibition.

Disadvantages of the agar plate technique will be discussed later (see page 146).

Spore suspensions were prepared by aseptically removing some spores from the aluminium foils on which they had been stored, and transferring them to sterile distilled water. Spore concentrations were always of the same order, about 20 to 200 X 10^4 spores per ml. With these concentrations the number which germinated varied from about 10 to 150 per plate.

0·1ml. of spore suspension was spread uniformly over the surface of each plate, and the plates were left to dry at 30°C. overnight.

Malt extract agar was used for all of the experiments, except the first one in which the following two media were also used. Hagem malt agar contains glucose - 5g., NH_4Cl - 0·25g., $MgSO_4.7H_2O$ - 0·25g., KH_2PO_4 - 0·25g.,

malt extract (Oxoid) - 5g., $FeSO_4.7H_2O$ - 5mg., agar (Davis, type P) - 15g. and distilled water to 1 litre. Complete agar is that used by Pontecorvo (1953), and contains a wide range of organic suplements.

Rhodotorula rubra, a non-spreading yeast, was used as a source of the activator which stimulates spore germination. It is closely related to Rhodotorula mucylaginosa var. sanguinea, the yeast used by M. Fries (1943). The culture, which was maintained on malt extract agar at 25°C., was subcultured every 6 weeks.

Agar plates, inoculated with spores, were treated with the yeast by inoculating at 5 points.

Rhodotorula rubra culture filtrate was prepared from shake cultures in 500ml. conical flasks, each containing 100ml. of 1·5 per cent. malt extract medium. Tho culture filtrate was collected after 7 days, concentrated at 40°C. to the equivalent of 20mg. dry weight of yeast per ml., and finally sterilized by micropore filtration. 0·1ml. of this culture filtrate was placed in a well cut from an agar plate.

In experiments with excised tomato roots 5 sectors were placed on an inoculated agar plate. 12 day old aseptically grown seedlings of Pinus sylvestris and Larix decidua were used, 3 per plate, in root experiments. Pinus sylvestris seeds were sterilized and germinated as previously described (see page 36). Seeds of Larix decidua, obtained from Thompson and Morgan Ltd of Ipswich, were sterilized by

treating first with 0•1 per cent. mercuric chloride plus a trace of Tween 80 for 2 minutes, and then washing in 6 changes of sterile distilled water. They were then further treated with 0•1 per cent. silver nitrate plus a trace of Tween 80 for one minute, and again washed in 6 changes of sterile distilled water. The seeds were germinated on 0•5 per cent. malt extract agar.

Excised tomato root exudate was prepared as previously described (see page 46). 0•1ml. of exudate was placed in a well cut from an agar plate.

Soil extracts were prepared from samples collected in Silwood Park, from directly under sporophores of Boletus elegans and Boletus bovinus. 9•5cm. Buchner funnels were fitted with Whatman No. 1 filter papers, and the soils were packed lightly on top. Butter muslin was then tied over the top of each funnel, and 100ml. of distilled water was allowed to drain through the soil. This same water was recirculated through the soil many times over a period of 2 days. Finally the extracts were concentrated at 40°C. to the equivalent of 2g. dry weight of soil per ml. The moisture contents of the soils were 48•3 per cent. (Boletus elegans soil) and 73•5 per cent. (Boletus bovinus soil). 0•1ml. of extract was placed in a well cut from an agar plate.

Interaction experiments.

Details of cultures of non-mycorrhizal Hymenomycetes used in these experiments are given in Table 3. All grow satisfactorally on malt extract agar.

TABLE 3. The non-mycorrhizal Hymenomycetes.

Species	Origin
Armillaria mellea	Bedford College
Clitocybe infundibuliformis	Baarn
Fomes sp.	Bedford College
Marasmius alliaceus	Baarn
Hypholoma fasciculare	"
Piptoporus betulinus	Bedford College
Trametes versicolor	Baarn

Soil saprophytic fungi were isolated from the soil samples previously used for the preparation of soil extracts (see page 53). Soil dilution plates were prepared using peptone-dextrose agar containing 30 p.p.m. rose bengal and 30 p.p.m. streptomycin (Martin, 1950). Plates treated with 5 and 50mg. fresh weight of soil gave the most satisfactory results. Control plates, i.e. peptone-dextrose agar alone, indicated that the method of isolation eliminated no

commonly occuring soil fungi. Two of the most frequently isolated species, a Penicillium species and a Trichoderma species, were selected for use in the experiments.

All experiments were conducted on malt extract agar, and the fungi were inoculated in pairs as shown in Fig. 6. Two replicate plates were set up for each treatment.

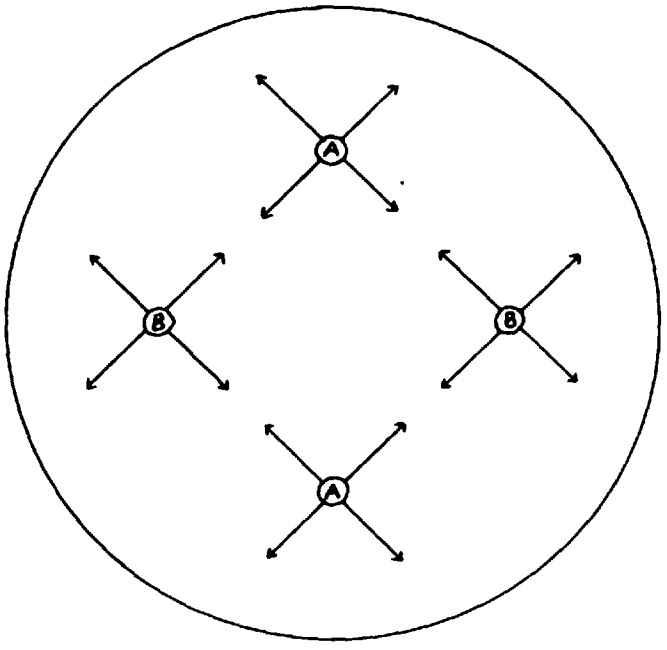


Fig. 6. Method for inoculation of interaction plates. A and B are the inocula of the two organisms and the arrows indicate the diameters measured.

RESULTS

Vigour and infectivity of the fungi.

In order to compare the vigour of the various isolates, the linear growth rates on malt extract agar were measured, both at the time of isolation (see page 27), and also at the end of the investigations. All rates are of growth in the log phase.

TABLE 4. Growth rates on malt extract agar in mm./week.

Fungus	Isolate no.	Growth at isolation	Growth, July, 1964	Growth (oil), July, 1964
B. elegans	1	7	9	7
" "	52	4	4	-
" "	63	4	5	-
" "	67	7	11	-
" "	77	6	6	-
" "	81	5	7	-
B. luteus	19	9	15	10
" "	44	7	8	-
" "	74	5	8	-
" "	79	10	10	-
" "	83	11	10	-
B. bovinus	42	20	11	19
" "	70	17	16	-

Table 4 continued:

B. bovinus	73	18	7	-
" "	78	21	15	-
B. variegatus	50	9	6	10
"		10	8	-
	72	15	8	-
" "	82	13	8	-

Boletus bovinus is generally the most vigourous, followed by Boletus luteus and Boletus variegatus, and finally Boletus elegans. Vigour decreased with time in Boletus variegatus and Boletus bovinus isolates, but seemed to increase in some isolates of Boletus elegans and Boletus luteus. Experimental cultures of the primary isolates were renewed periodically from the cultures kept under oil.

The growth rates of the primary isolates were also measured on the ionagar media.

TABLE 5. Growth rates on ionagar media in mm./week.

Fungus	Isolate no.	Growth on basic ionagar	Growth on maximum ionagar
B. elegans	1	9	9
B. luteus	19	21	20
B. bovinus	42	13	10
B. variegatus	50	13	15

Although the growth rates on the two media were approximately equal, there was a distinct lag of about 48 hours on the maximum ionagar for all four fungi.

Many pathogenic fungi, if kept in pure culture for long periods of time, tend to lose their pathogenicity. The same could be true of symbiotic fungi. It is known that some mycorrhizal fungi tend to change with respect to vigour, production of fruiting initials and morphological characteristics, e.g. production of clamp connections. This is true of some of the present isolates. It was therefore considered advisable to test these isolates for their ability to produce mycorrhizas in vitro with their normal hosts.

Only three isolates gave positive results.

TABLE 6. In vitro synthesis of mycorrhizas.

Fungus	Isolate no.	Host and time of inoculation	Time of sampling
B. elegans	1	larch, 84 days	140 days
" "	63	" " "	" "
B. luteus	19	pine, 82 days	126 days

Negative results were obtained with all other isolates. In the three successful cases mycelial suspensions and a medium pH of 7·2 were used.

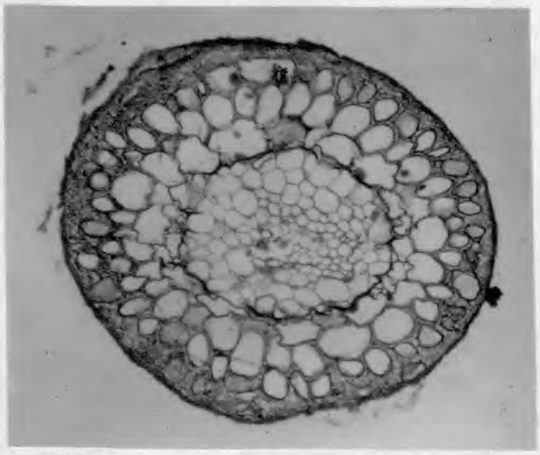
All three mycorrhizas successfully synthesized were of the simple, unbranched type. The one between Boletus luteus (19) and Pinus sylvestris was examined in more detail (Fig. 7). A typical sheath was present, and also a Hartig net consisting of intercellular hyphae passing into the root cortex, and reaching to within one or two cell layers of the endodermis.

Nutrition of the fungi.

Temperature optima for the primary isolates were determined on maximum ionagar by measuring growth rates during the log phase of growth. Temperature-growth curves are shown in Fig. 8. Growth was best at 25°C. for all four isolates.

The effect of pH on growth of the primary isolates was determined in maximum medium using Sorensen's phosphate buffers. The buffers were made up as follows.

pH	M/15 KH_2PO_4	M/15 Na_2HPO_4	M/10 HCl
4·5	146ml.	-	4ml.
5·0	149·5ml.	0·5ml.	-
5·8	131·5ml.	18·5ml.	-
7·0	55ml.	95ml.	-



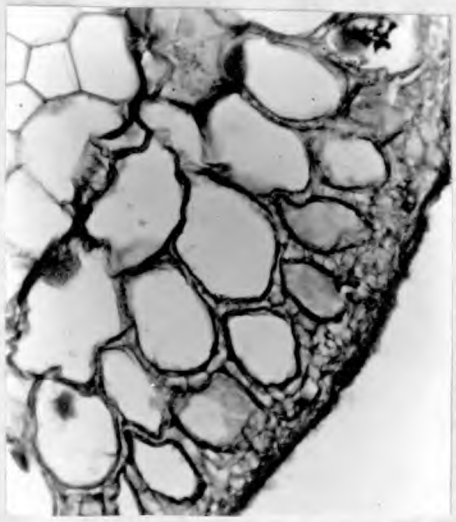


Fig. 7. Synthetic mycorrhiza between Pinus sylvestris and Boletus luteus (19). Mycorrhizal short roots (top left), L.S. of short root (top right), T.S. of short root near tip (bottom left) and T.S. showing details of fungal sheath and Hartig Net (bottom right).

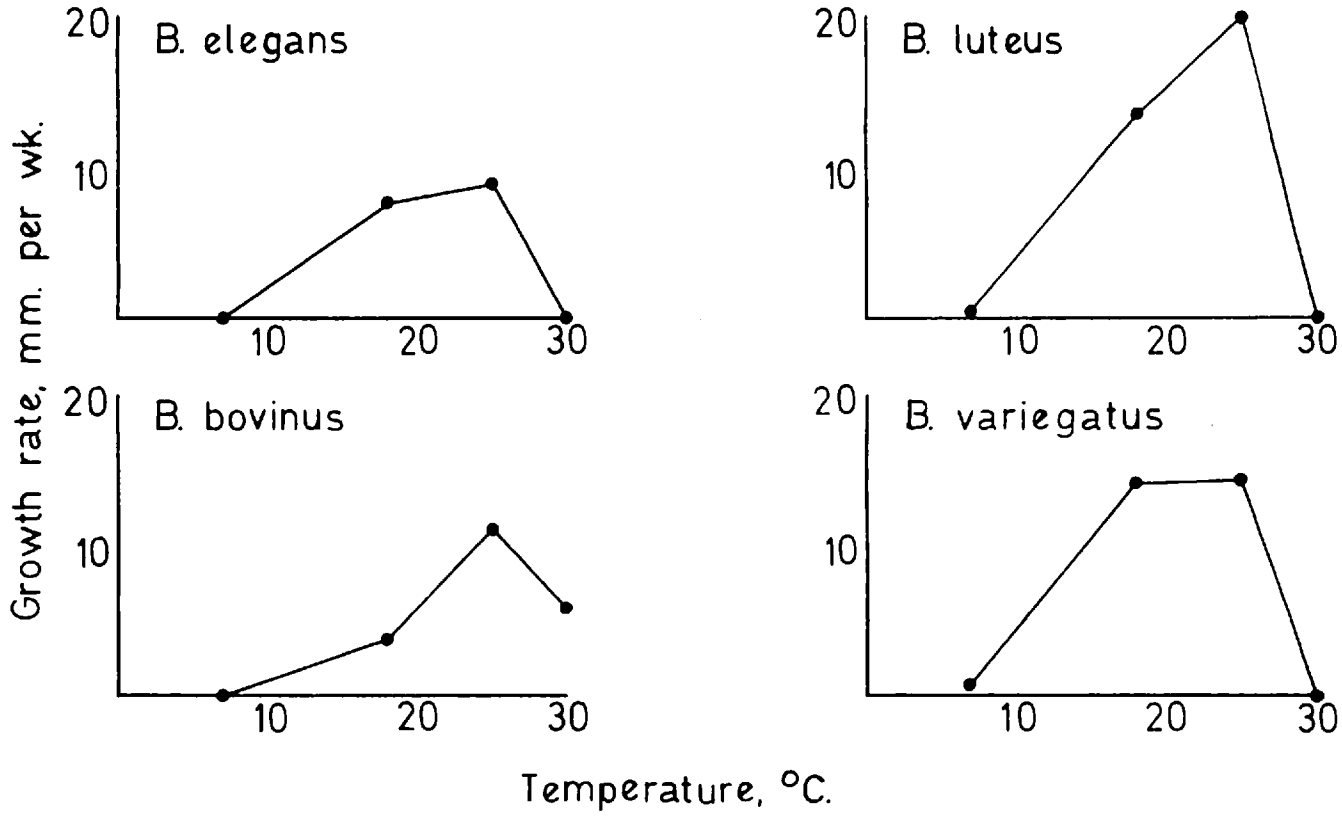


Fig. 8. Temperature-growth curves for the primary isolates.

6ml. of buffer was included in 20ml. of maximum medium (less the KH_2PO_4), and was added aseptically after autoclaving. pH-growth curves are shown in Fig. 9. All four isolates grew well over the pH range 4·5 to 5·8, the optimum in each case being about 5·0.

The ability of the primary isolates to utilize various simple sugars and polysaccherides as carbon and energy sources, was determined in basic medium, the glucose of which had been replaced by one of a range of carbohydrates.

TABLE 7. Growth with various simple sugars.

	Dry weight in mg.			
Sugar (2%)	B. elegans	B. luteus	B. bovinus	B. variegatus
nil	0·6 (0·1)	0·8 (0·1)	0·4 (0·1)	0·8 (0·2)
Glucose	7·3 (0·6)	8·6 (0·5)	9·0 (0·5)	10·1 (0·6)
Fructose	2·2 (0·2)	5·4 (0·2)	3·0 (0·3)	1·6 (0·4)
Galactose	2·9 (0·4)	4·3 (0·4)	0·8 (0·1)	0·1
Mannose	5·8 (0·3)	5·6 (0·3)	3·6 (0·1)	8·3 (0·3)
Xylose	1·5 (0·4)	1·5 (0·1)	1·2 (0·1)	1·9 (0·1)
Mannitol	3·1 (0·4)	4·9 (0·4)	2·4 (0·2)	4·3 (0·6)
Maltose	6·2 (0·3)	4·3 (0·3)	2·8 (0·1)	5·8 (0·4)
Sucrose	4·4 (0·4)	5·0 (0·4)	3·5 (0·4)	4·0 (0·2)
Acetate	0·9 (0·1)	1·0 (0·1)	0·7 (0·1)	1·1 (0·1)
Malate	1.7 (0·1)	1·5 (0·1)	0·8 (0·1)	1·5 (0·1)

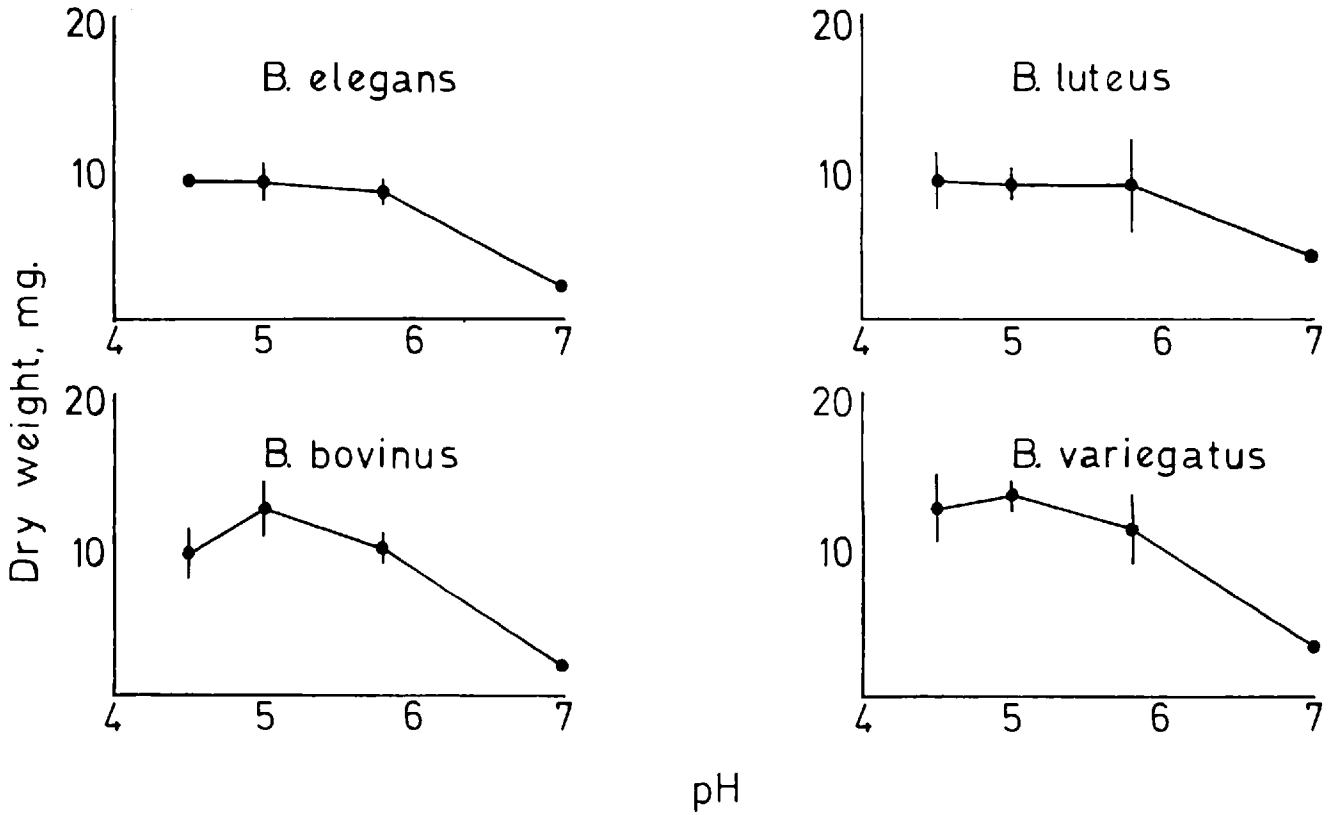


Fig. 9. pH-growth curves for the primary isolates.

Inall cases growth was best with glucose. Mannose, maltose and sucrose were generally good, whereas fructose, galactose, xylose, acetate and malate were generally poor. The relative growth of the fungi with the various sugars is shown in Fig. 10. Despite the fact that leached inocula were used in this and in all other carbohydrate experiments some growth occured in the controls.

TABLE 8. Growth with various polysaccherides.

	Dry weight in mg.			
Sugar (1%)	B. elegans	B. luteus	B. bovinus	B. variegatus
nil	0·6 (0·1)	0·8 (0·1)	0·4 (0·1)	0·8(0·2)
Glucose(2%)	7·3 (0·6)	8·6 (0·5)	9·0 (0·5)	10.1 (0·6)
Starch(2%)	0·7 (0·1)	1·2 (0·1)	1·1 (0·1)	0·7 (0·1)
Pectin	0·9 (0·1)	1·3 (0·1)	1·2 (0·1)	0·8 (0·2)
Polypectate	1·5 (0·3)	1·9 (0.2)	1·3 (0·1)	1·2 (0·1)
C.M.C.	0·7 (0·1)	0·5 (0·1)	0·4 (0·1)	0·1

Pectin and polypectate supported slight growth of Boletus luteus ($P<0.05$), while starch, pectin and polypectate supported slight growth of Boletus bovinus ($P<0.01$). Carboxymethyl-cellulose inhibited the growth of Boletus variegatus ($P<0.01$). The relative growth of the fungi with the various

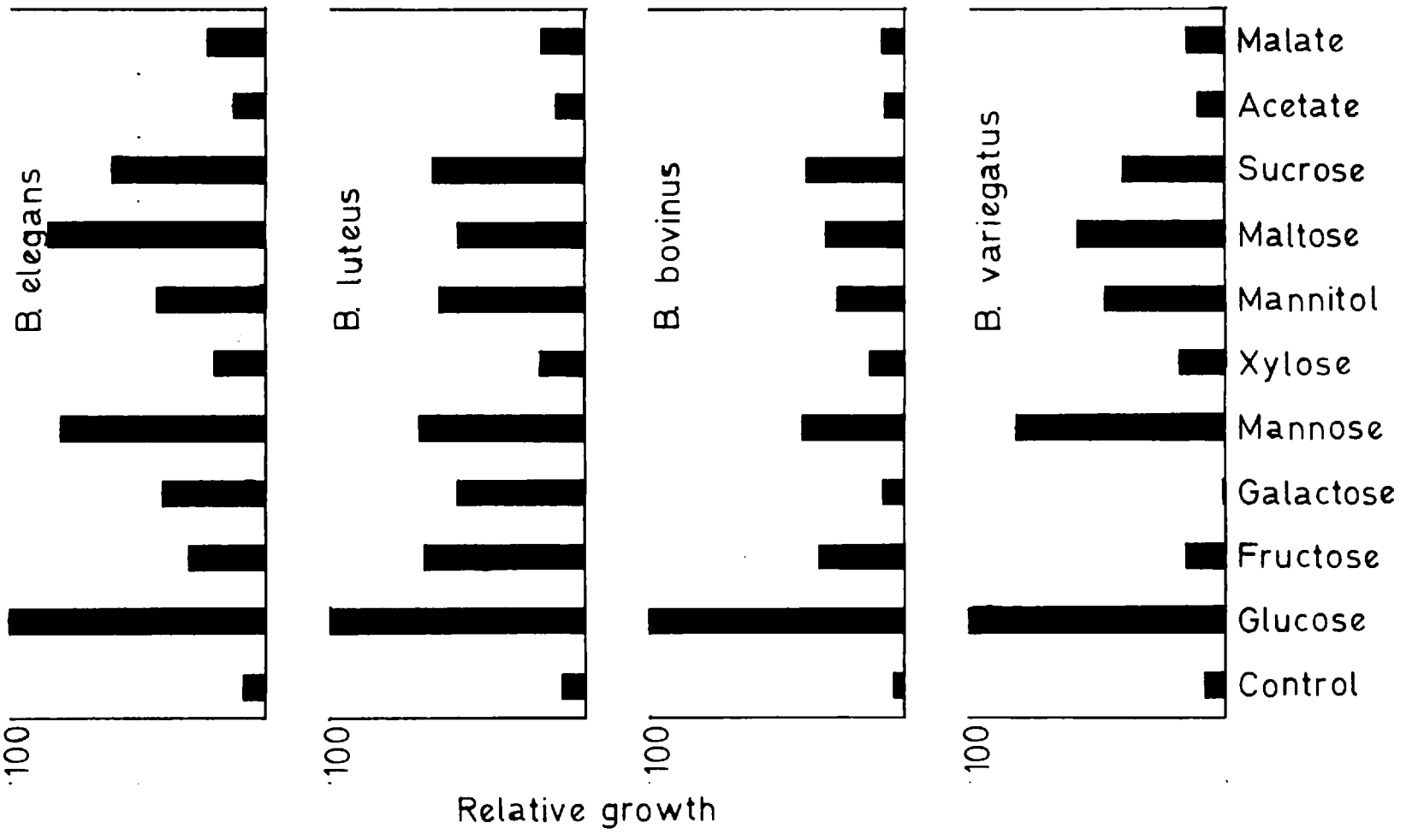


Fig. 10. Relative growth with simple sugars.

polysaccherides is shown in Fig. 11.

Growth on two insoluble substrates, wood cellulose and Esparto hemicellulose, was measured on basic ionagar. The substrates were dispersed uniformly through the agar.

TABLE 9. Growth with insoluble substrates.

	Radial growth in one week in mm.			
Sugar(1%)	B. elegans	B. luteus	B. bovinus	B. variegatus
Glucose(2%)	7	18	14	12
Wood cellulose	1	0	1	1
Hemicellulose	0	0	0	0

Very slight but insignificant growth occured with the wood cellulose for three of the isolates.

All polysaccherides were checked to see if steaming caused any breakdown. After steaming solutions or suspensions were dialysed, the dialysates were concentrated and chromatograms were run in iso-propanol-water. Aniline-diphenylamine revealed no simple sugars in excess of 0·05 per cent.

A further experiment was done to see if the addition of a trace of glucose permitted improved utilization of any of the polysaccherides. The incubation period for this

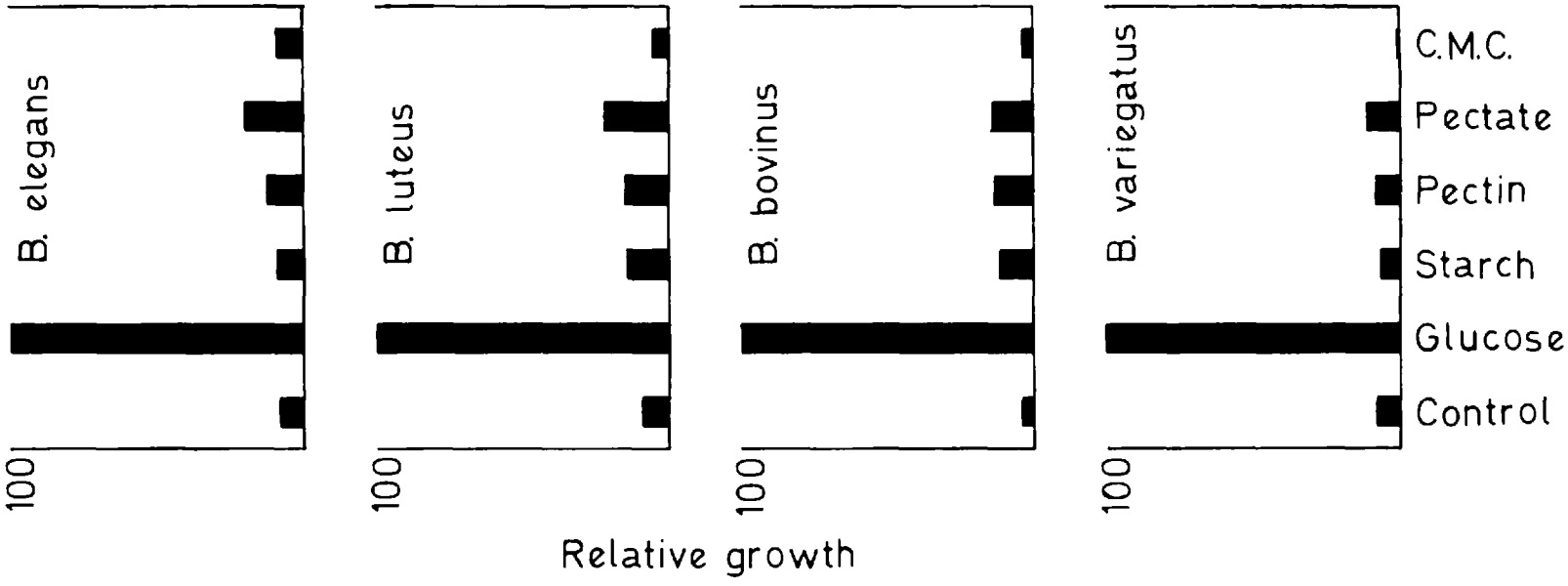


Fig. 11. Relative growth with polysaccherides.

experiment was 21 days.

TABLE 10. Growth with various polysaccherides.

Sugar (1%)	Dry weight in mg. B. elegans	B. variegatus
nil	0·7 (0·1)	0·9 (0·1)
Glucose (0·05%)	6·4 (0·1)	7·5 (0·1)
Starch (2%)	0·9 (0·1)	1·1 (0·1)
Starch (2%) + glucose (0·05%)	9·5 (0·2)	8·3 (0·1)
Pectin	1·3 (0·1)	1·5 (0·1)
Pectin + glucose (0·05%)	9·9 (0·3)	8·7 (0·1)
Polypectate	1·2 (0·1)	1·2 (0·1)
Polypectate + glucose (0·05%)	7·5 (0·3)	8·1 (0·4)
C.M.C.	0·9 (0.1)	03 (0·1)
C.M.C. + glucose (0·05%)	4·1 (0·3)	4·9 (0·1)

A trace of glucose did improve the utilization of starch and pectin for both fungi. On the other hand carboxy-methyl-cellulose reduced the growth of both fungi on glucose. An analysis of variance is given in Appendix C. The relative growth of the fungi with the various polysaccherides, with and without a trace of glucose, is shown in Fig. 12.

The growth of the primary isolates was compared on

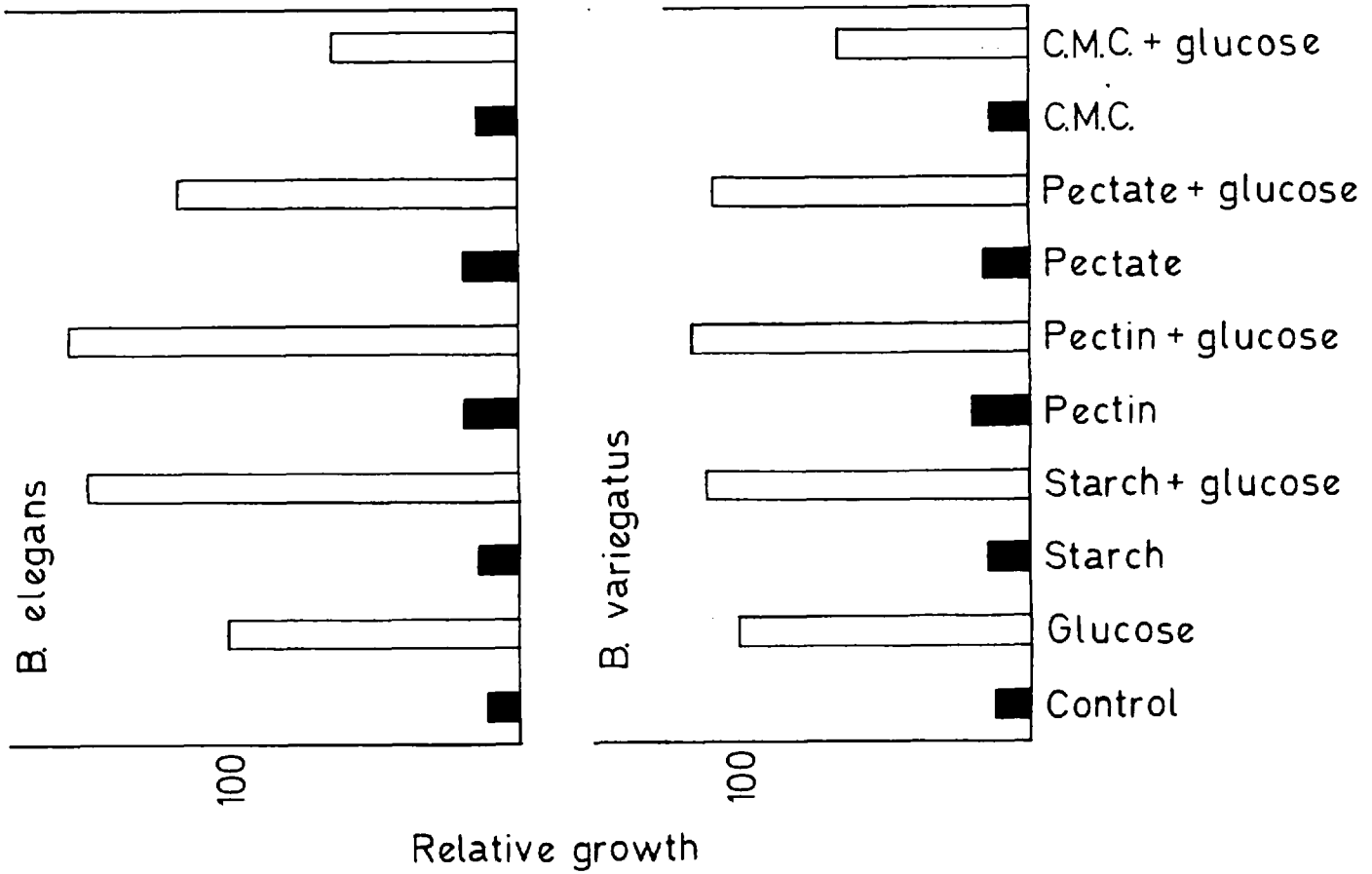


Fig. 12. Utilization of polysaccherides in the presence of a trace of glucose.

different nitrogen sources in basic medium.

TABLE 11. Growth with various nitrogen sources.

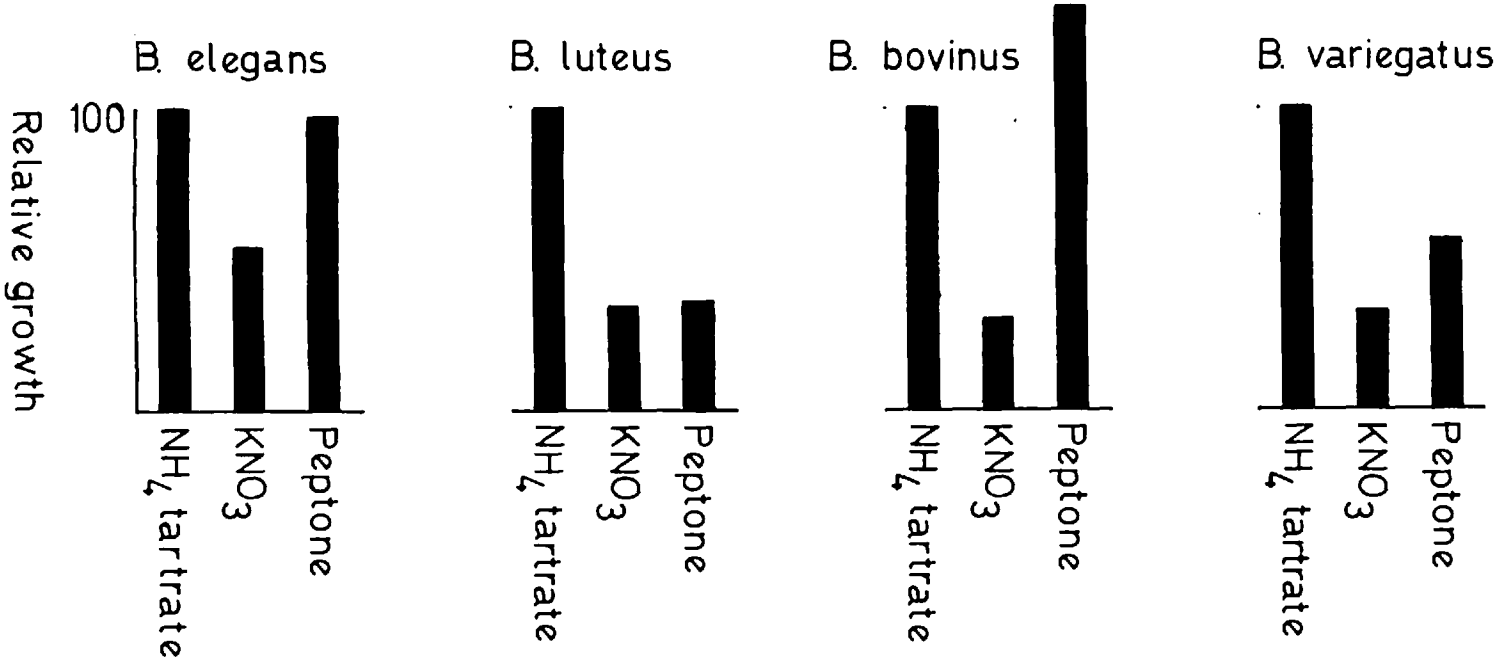
N source (0.0027M, N)	Dry weight in mg. B. elegans	B. luteus	B. bovinus	B. variegatus
NH_4 tartrate	7.3 (0.3)	8.6 (0.5)	9.2 (0.5)	10.1 (0.6)
KNO_3	3.9 (0.6)	2.9 (0.1)	2.8 (0.2)	3.3 (0.5)
Peptone	7.1 (0.3)	3.1 (0.4)	12.3 (0.4)	5.7 (0.3)

Growth with ammonium tartrate was generally superior, but Boletus elegans grew equally well on peptone and Boletus bovinus grew better on peptone ($P<0.05$). The relative growth of the fungi with the various nitrogen sources is shown in Fig. 13.

Because of the fact that anumber of fungi, including a few Hymenomycetes, are known to require calcium, the effect of this nutrient on the growth of the primary isolates in maximum medium was examined. The results are shown in Fig. 14. Only Boletus elegans was stimulated by calcium. High concentrations were generally inhibitory.

The effect of the amino acid and vitamin mixtures on the growth of the primary isolates was also examined. Leached inocula were used.

Fig. 13. Relative growth with various nitrogen sources.



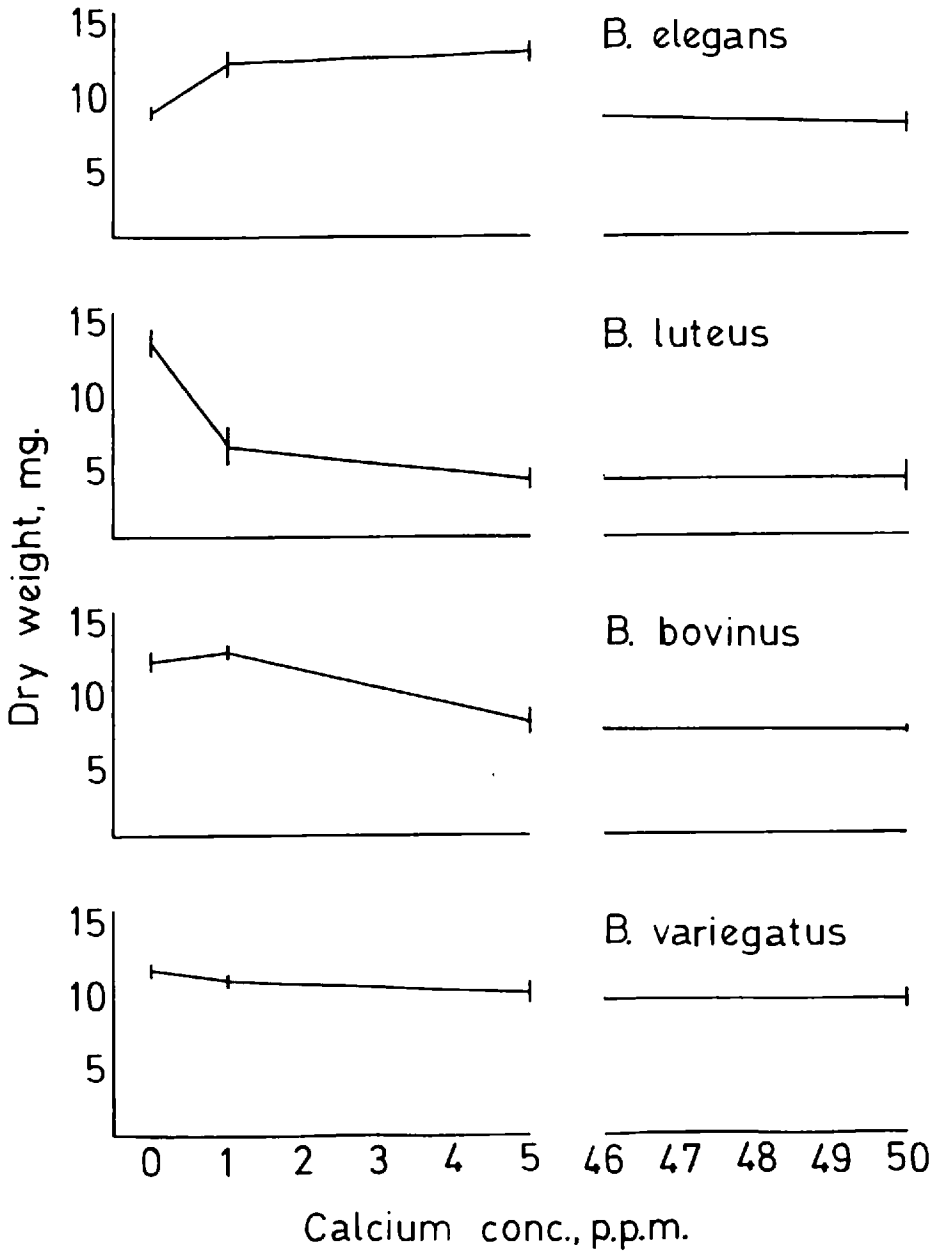


Fig. 14. Growth of primary isolates with calcium.

TABLE 12. Growth with amino acids and vitamins.

Medium	Dry weight in mg. B. elegans	B. luteus	B. bovinus	B. variegatus
Basic - thiamin	3·0 (0·3)	3·3 (0·2)	4·1 (0·2)	3·4 (0·2)
Basic - thiamin + amino acids	7.0 (0·3)	7·0 (0·1)	4·6 (0·3)	5·3 (0·2)
Basic + vitamins	7·6 (0·2)	11·2 (0·2)	7·1 (0·2)	10·0 (0·2)
Maximum	10·4 (0·3)	11·4 (0·3)	10·1 (0·3)	11·6 (0·3)

Both the amino acids and the vitamins stimulated the growth of the fungi ($P<0.01$), except for the amino acids in the case of Boletus bovinus. Growth in the maximum medium indicated that the amino acids and the vitamins interacted negatively in the case of Boletus luteus, and positively in the case of Boletus bovinus (see Appendix C for analysis of variance). The relative growth of the fungi is shown in Fig. 15.

The effect of the amino acids added individually to a medium consisting of the basic medium plus the vitamin mixture was tested. The acids were added at the concentrations used in the amino acid mixture.

Fig. 15. Relative growth with amino acids and vitamins.

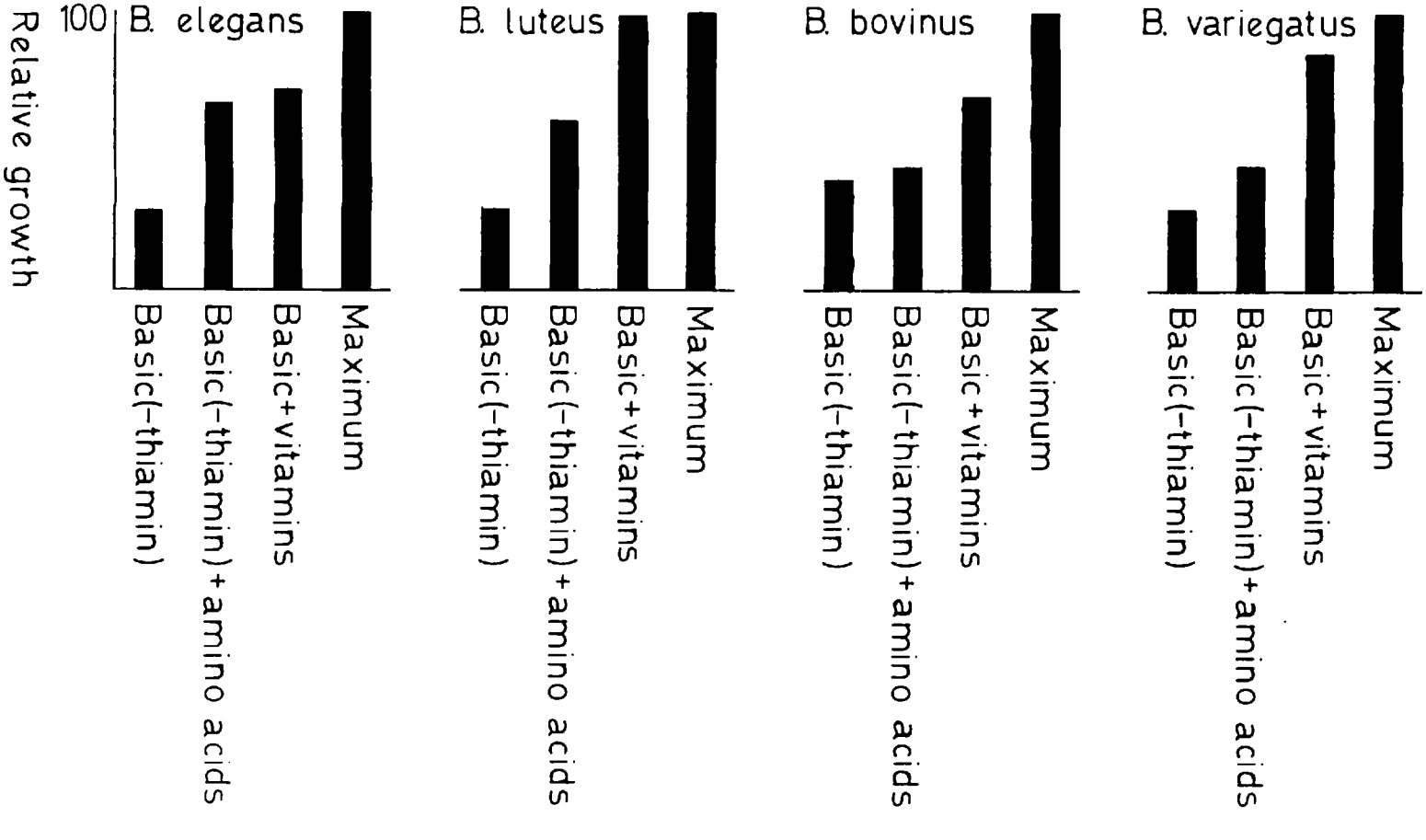


TABLE 13. Growth with individual amino acids.

Amino acid added	Dry weight in mg.			
	B. elegans	B. luteus	B. bovinus	B. variegatus
nil	10·3 (0·3)	11·6 (0·3)	8·5 (0·3)	9·4 (0·3)
Mixture	14.1 (0·3)	14·5 (0·1)	12·1 (0·3)	11·5 (0·4)
Glutamic acid	11·1 (0·2)	14·1 (0·3)	10·9 (0·3)	11·0 (0·3)
Proline	5·5 (0·2)	4·0 (0·2)	5·8 (0·3)	5·1 (0·3)
Hydroxy-proline	10·2 (0·6)	5·2 (0·2)	8·2 (0·3)	7·8 (0·5)
Tryptophane	6·9 (0·5)	9·6 (0·2)	7·3 (0·2)	7·8 (0·7)
Tyrosine	1·1 (0·1)	5·7 (0·2)	4·8 (0·2)	0·5 (0·1)
Phenylalanine	0·9 (0·1)	2·5 (0·2)	4·0 (0·3)	1·8 (0·3)
Aspartic acid	9·9 (0·2)	10·5 (0·3)	6·5 (0·4)	10·3 (0·4)
Arginine	9·2 (0·2)	5·9 (0·2)	9·3 (0·4)	12·6 (1·0)
Histidine	6·7 (0·3)	9·8 (0·3)	10·3 (0·3)	4·6 (0·3)
Lysine	6·6 (0·3)	11·3 (0·2)	6·7 (0·1)	8·8 (0·5)

Glutamic acid stimulated the growth of Boletus variegatus ($P<0·05$), and of Boletus luteus and Boletus bovinus ($P<0·01$). Several acids inhibited growth, especially proline, tyrosine, phenylalanine and histidine. The relative growth of the fungi with the individual acids is shown in Fig. 16.

In a further experiment certain amino acids were

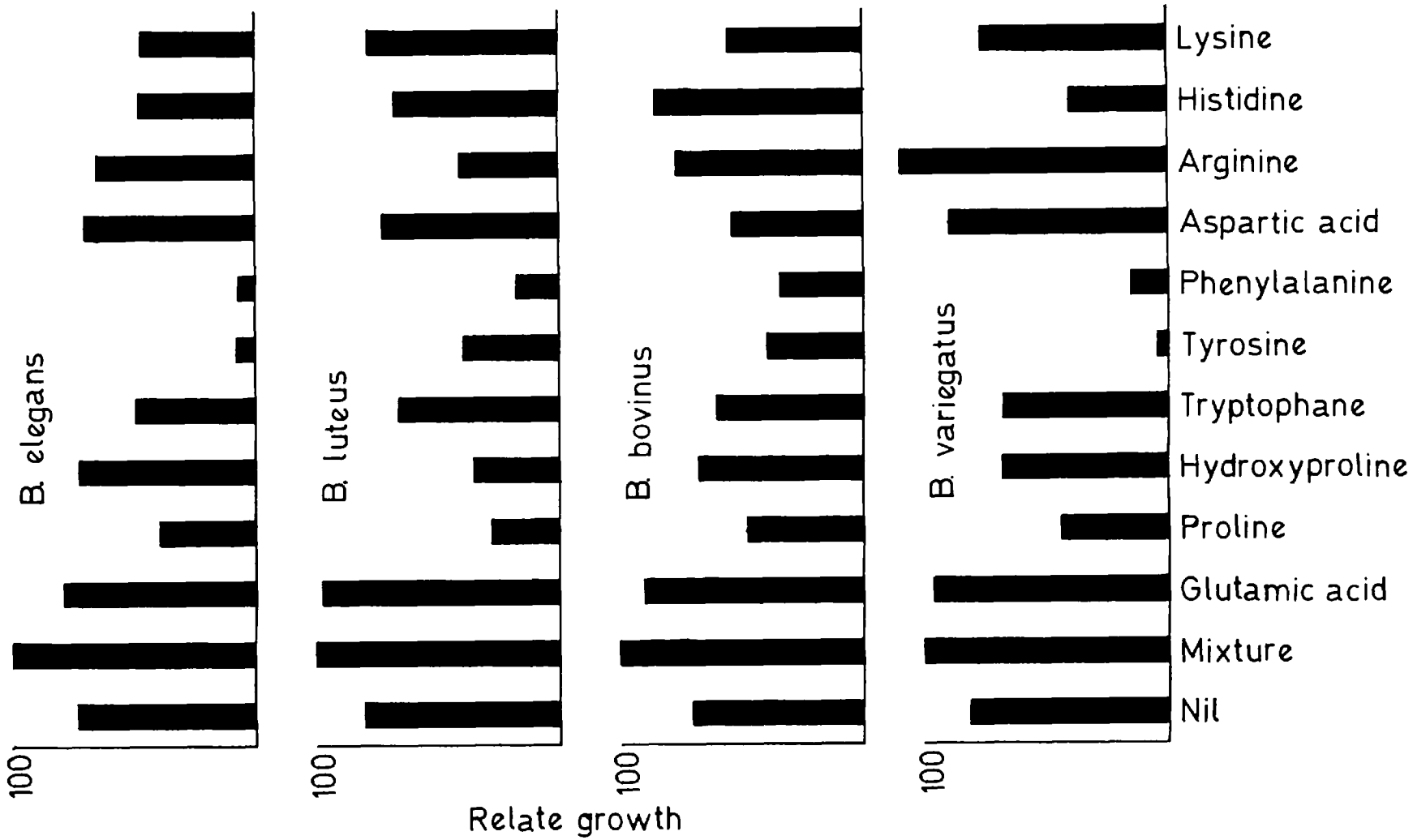


Fig. 16. Relative growth with individual amino acids.

omitted from the maximum medium.

TABLE 14. Growth with certain amino acids omitted.

Amino acid omitted	Dry weight in mg.			
	B. elegans	B. luteus	B. bovinus	B. variegatus
Mixture	6·9 (0·1)	10·7 (0·3)	10·0 (0·3)	6·0 (0·1)
nil	11.2 (0.4)	12·3 (0.1)	11·4 (0·2)	11.0 (0.4)
Glutamic acid	11·6 (0·3)	12·3 (0·3)	9·5 (0·2)	11·4 (0·3)
Proline	14·3 (0.3)	12·3 (0·2)	10·8 (0.2)	12.5 (0·2)
Tyrosine	12·5 (0·2)	17·0 (0.6)	12·4 (0.1)	12·0 (0·2)
Phenylalanine	11·9 (0·2)	14·1 (0·3)	13·9 (0·4)	13·3 (0·1)
Aspartic acid	11·7 (0·3)	9·5 (0·4)	10·7 (0·1)	13·8 (0·1)
Arginine	13·2 (0·3)	10.7 (0·3)	10·2 (0·2)	12.8 (0·3)
Histidine	9·9 (0·3)	8·0 (0·3)	11·5 (0·2)	11·2 (0·2)

Although the results generally conform with those of the previous experiment, none of the effects are of the same magnitude. A requirement for glutamic acid is apparent only for Boletus bovinus ($P<0·01$). Removal of aspartic acid, arginine or histidine from the amino acid mixture resulted in reduced growth of Boletus luteus ($P<0·01$). Boletus bovinus responded similarly to the removal of aspartic acid or arginine ($P<0·05$). Boletus elegans was stimulated by the

removal of proline ($P<0.01$) or arginine ($P<0.05$), Boletus luteus and Boletus bovinus by the removal of tyrosine ($P<0.01$ and <0.05) or phenylalanine ($P<0.01$) and Boletus variegatus by the removal of, proline ($P<0.05$), phenylalanine ($P<0.01$), aspartic acid ($P<0.01$) or arginine ($P<0.05$). The relative growth of the fungi is shown in Fig. 17.

The concentrations of the amino acids were too low to contribute to the nitrogen pool of the medium to any significant extent. However with glutamic acid this was not so, and therefore an experiment was done to compare glutamic acid and ammonium tartrate as nitrogen sources, in a medium consisting of the basic medium plus the vitamin mixture. The results are shown in Fig. 18. Glutamate supported only slight growth at all concentrations, and there was no interaction between the glutamate and the ammonium salt when these were present together in the medium.

It is possible that some of the amino acids are inhibitory because they are transformed into toxic compounds during autoclaving. This was tested by autoclaving solutions of proline, tryptophane, tyrosine, phenylalanine, arginine and histidine (1mg. per ml.) and running chromatograms in n-butanol-acetic acid-water. The acids remained unchanged as indicated by dipping the papers in ninhydrin, isatin, aniline-xylose and ferric chloride-potassium ferricyanide reagent.

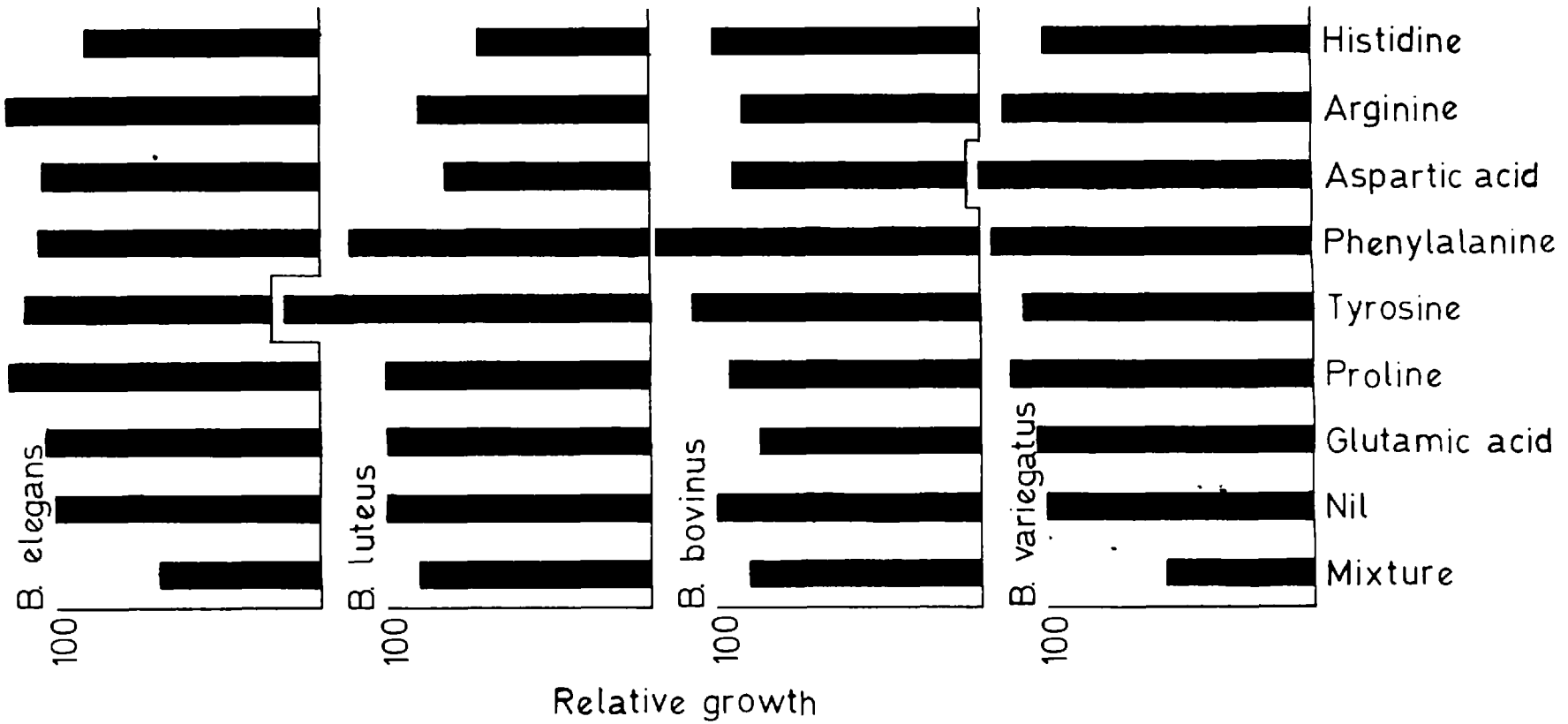


Fig. 17. Relative growth with single amino acids omitted from the maximum medium.

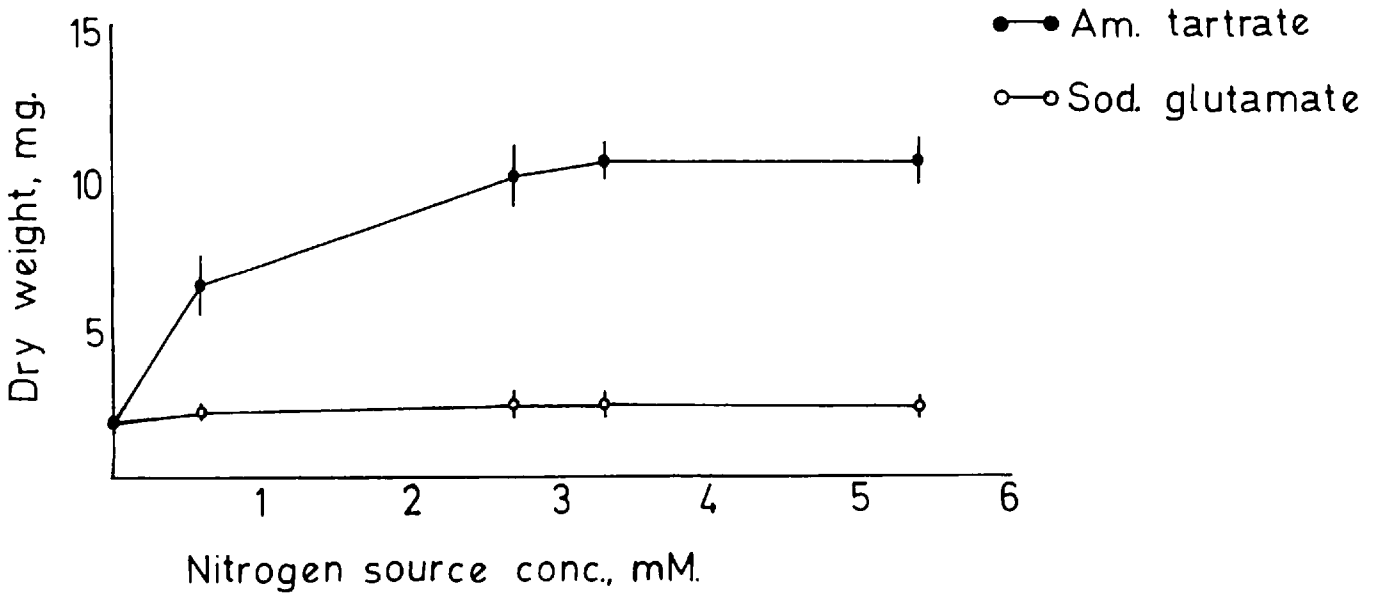


Fig. 18. Growth of Boletus variegatus (50) with ammonium and glutamate as nitrogen sources.

The response of the fungi to the vitamin mixture was further analysed. Leached inocula were used.

TABLE 15. Growth with vitamins.

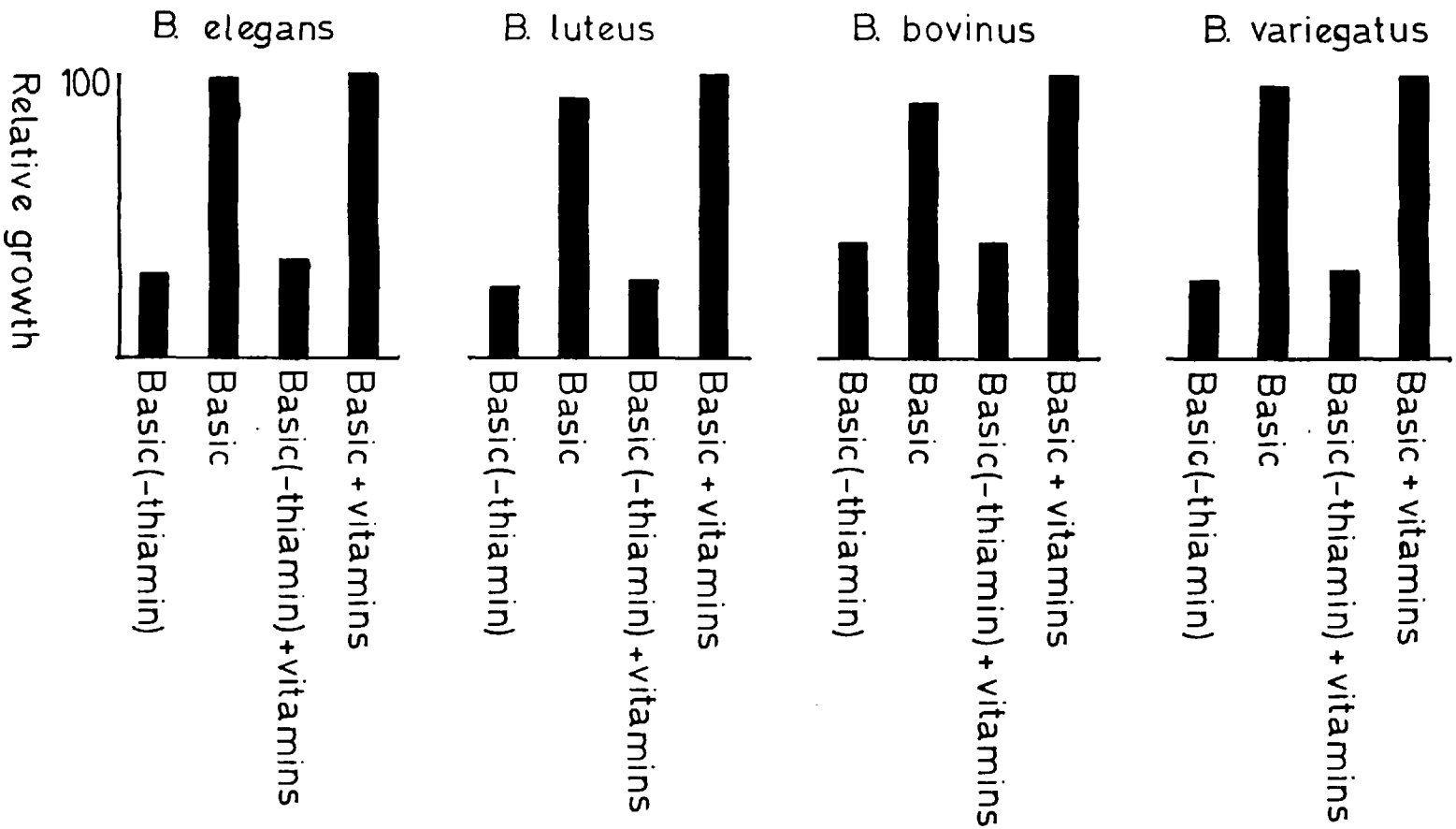
Medium	Dry weight in mg. B. elegans	B. luteus	B. bovinus	B. variegatus
Basic - thiamin	2·2 (0·3)	2·8 (0·2)	3·2 (0·2)	2·9 (0·2)
Basic	7·2 (0·1)	10·1 (0·4)	7·0 (0·1)	10·1 (0·2)
Basic - thiamin + vitamins	2·5 (0·3)	3·0 (0·2)	3·2 (0·3)	3·3 (0·3)
Basic + vitamins	7·3 (0·2)	11·0 (0·2)	7·8 (0·2)	10·5 (0·2)

Thiamin stimulated the growth of all four fungi ($P<0·01$). The vitamin mixture did not stimulate growth, and there was no interaction between thiamin and the vitamin mixture for any of the fungi.(see Appendix C for analysis of variance). The relative growth of the fungi with vitamins is shown in Fig. 19.

Experiments with roots and root exudates.

The results of experiments with excised tomato roots are given in Tables 16, 17 and 18. Non-sieved mycelial suspensions were used in the first and second experiments, and sieved mycelial suspensions in the third. The incubation

Fig. 19. Relative growth with thiamin and other vitamins.



period for the first two experiments was 9 days and for the third 12 days

TABLE 16. Effect of excised tomato roots (Clone C) on growth.

Medium	Dry weight in mg. B. elegans	B. luteus	B. bovinus	B. variegatus
Basic	2·2 (0·3)	3·7 (0·2)	7·1 (0·3)	0·4 (0·1)
Basic + root	8·1 (0·5)	30·4 (1·0)	21·8 (0·5)	21·3 (1·5)
Maximum	1·1 (0·3)	4·2 (0·3)	7·2 (0·3)	4·9 (0·4)
Maximum + root	10·9 (0·6)	67·4 (2·6)	53·7 (4·3)	48·7 (6·0)

TABLE 17. Effect of excised tomato roots (Clone C) on growth

Medium	Dry weight in mg. B. luteus (19)	B. luteus (44)	B. bovinus
Basic	3·9 (0·3)	0·4 (0·1)	15·6 (0·3)
Basic + root	35·0 (2·6)	17·4 (1·7)	42·4 (1·2)
Maximum	3·5 (0·2)	1·8 (0·4)	20·2 (3·1)
Maximum + root	54·1 (3·2)	26·2 (1·0)	90·0 (3·4)

TABLE 18. Effect of excised tomato roots (Clone C) on growth

Medium	Dry weight in mg.			
	B. elegans	B. luteus	B. bovinus	B. variegatus
Maximum	1·3 (0·1)	0·3 (0·1)	0·1	0·1
Maximum + root	12·5 (1·2)	55·6 (1·5)	101·9 (2·7)	59·0 (3.7)
Maximum + killed root	0·9 (0·2)	0·1	1·0 (0·1)	0·4 (0·2)

The fungi were stimulated considerably by the roots, both in basic and maximum medium, and there was a marked positive interaction between the maximum medium and the roots in all cases (see Appendix C for analysis of variance). Killed roots were ineffective, except for Boletus bovinus, which was stimulated slightly. The relative growth of the fungi in these experiments is shown in Figs 20, 21 and 22.

Further experiments were carried out with all of the Boletus isolates, and also with a range of non-mycorrhizal Hymenomycetes, on maximum ionagar. Plates were inoculated with 20 microlitre drops of sieved mycelial suspensions. The results are given in Fig. 23. The Boletus isolates were stimulated by the roots, whereas the non-mycorrhizal Hymenomycetes were not. The growth of Marasmius alliaceus was inhibited by roots.

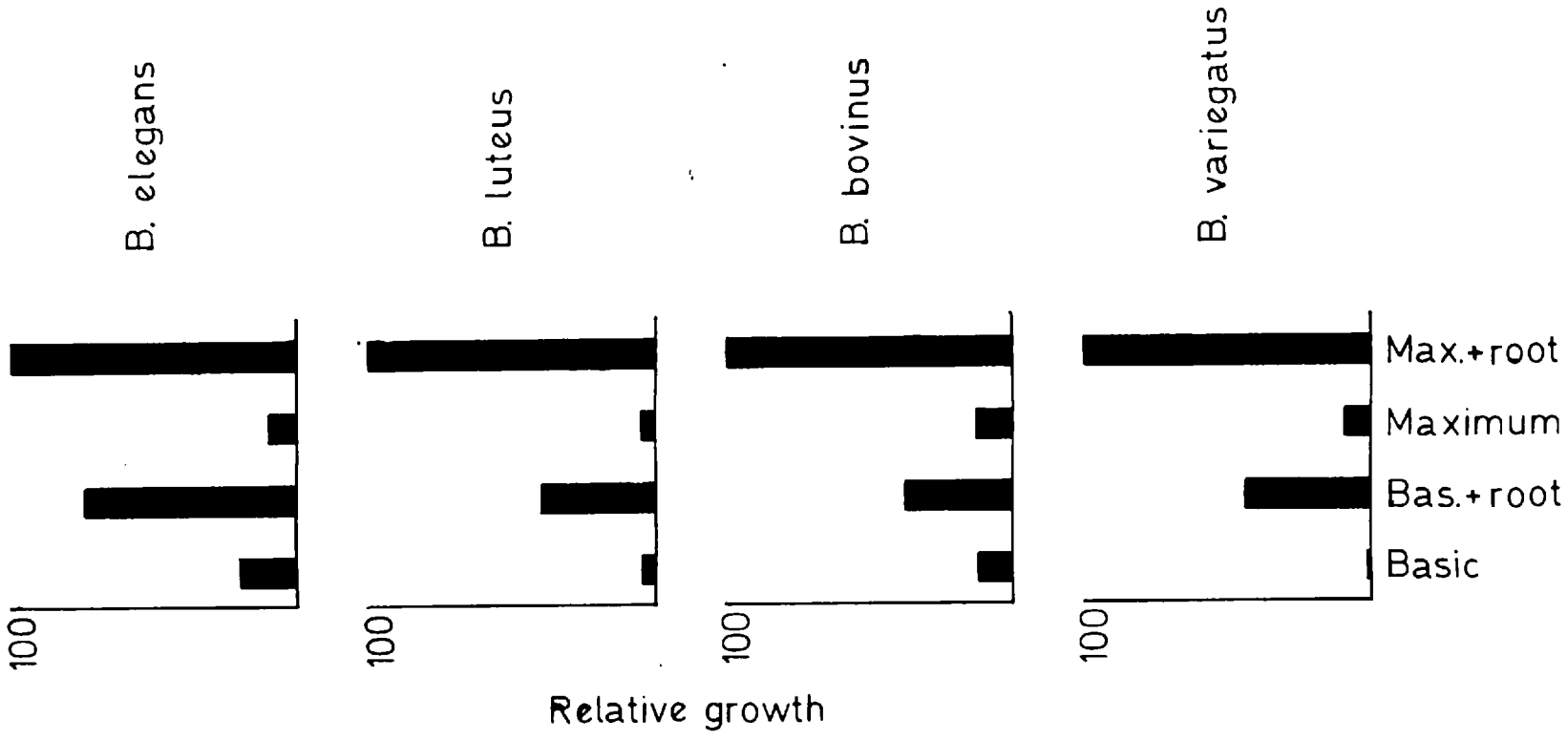


Fig. 20. Relative growth with and without excised tomato roots.

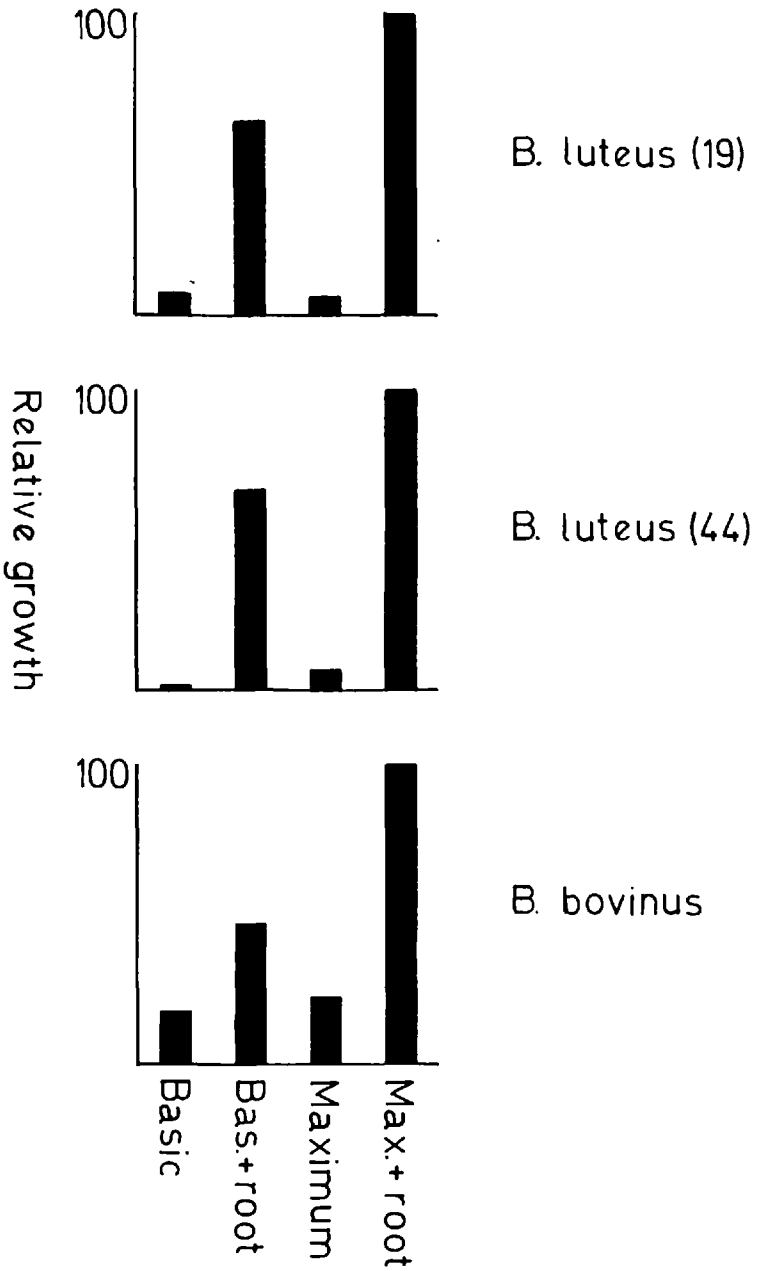


Fig. 21. Relative growth with and without excised tomato roots.

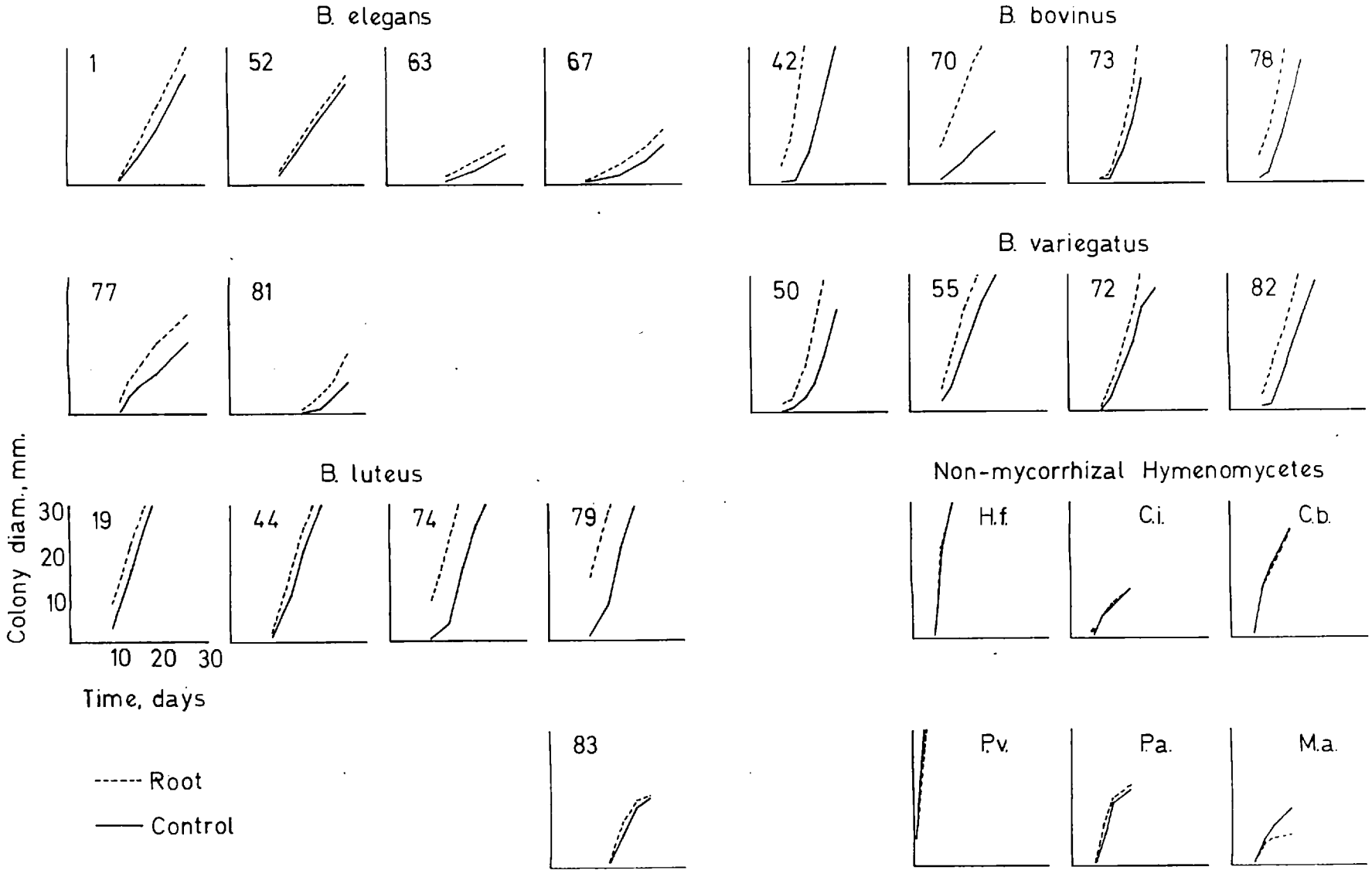


Fig. 23. Growth with and without excised tomato roots (Clone C). H.f. = Hypholoma fasciculare; C.i. = Clitocybe infundibuliformis; C.b. = Collybia butyracea; T.v. = Trametes versicolor; P.a. = Paxillus atromentosus, and M.a. = Marasmius alliaceus.

A single experiment was conducted with Boletus variegatus in which agar inocula were used.

TABLE 19. Effect of excised tomato roots (Clone C) on growth

Medium	Dry weight in mg.
Maximum	11·2 (0·9)
Maximum + root	23·8 (1.0)
Maximum + killed root	16·2 (0·4)

The fungus was stimulated by the roots ($P<0.01$). Killed roots also stimulated growth ($P<0.01$), but not to the same extent.

The effect of roots on growth was also demonstrated using the auxanographic technique (Fig. 24).

The results of an experiment with excised roots of Pinus sylvestris are given below. Non-sieved mycelial suspension was used and the incubation period was 9 days.

The stimulation of growth is comparable with that obtained with excised tomato roots, although the dry weight of a pine root was only 2·6mg. compared with the tomato root 7·5mg. Killed roots also stimulated growth ($P<0.01$), but not to the same extent as living roots.

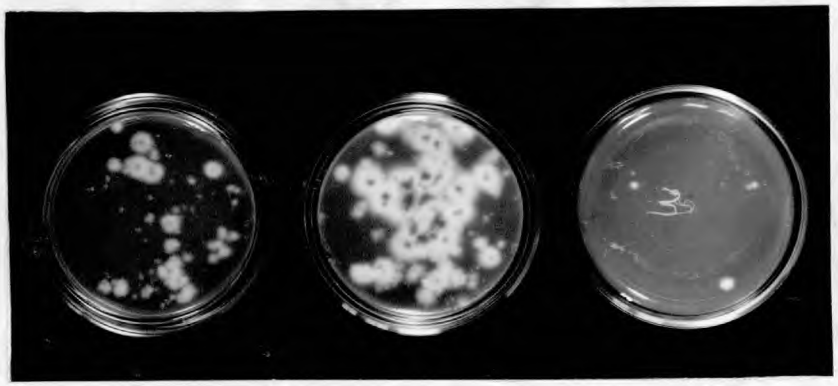


Fig. 24. Auxanographic technique. Growth of Boletus luteus - control (left), with excised tomato root sector, Clone C, (centre) and with killed root sector (right).

TABLE 20. The effect of excised pine roots on the growth of Boletus variegatus.

Medium	Dry weight in mg.
Maximum	4·5 (0·3)
Maximum + root	29·3 (1·4)
Maximum + killed root	8·5 (0·4)

Finally the results of an experiment in which aseptically grown tomato seedlings were used are given below. Sieved mycelial suspension was used and the incubation period was 14 days. Two seedlings, aged 10 days and having a combined root dry weight of approximately 12mg., were added to each flask, the roots only being immersed in the liquid.

TABLE 21. Effect of tomato seedling roots on the growth of Boletus variegatus.

Medium	Dry weight in mg.
Maximum	0·6 (0·1)
Maximum + seedlings	27·3 (1·2)

The fungus was stimulated by the seedling roots ($P<0.01$), but not to the same extent as by excised roots.

A preliminary experiment was conducted with root exudates collected in water and in dilute White's medium for 2 weeks. The incubation period was 16 days.

TABLE 22. Effect of excised tomato root exudate on the growth of Boletus variegatus.

Colony diameter in mm.

Medium	Exudate collected in:- Water	White's/100	White's/10
Max. ionagar	13.3 (0.5)	13.5 (0.3)	14.8 (0.5)
Max. ionagar + exudate	22.3 (0.5)	21.5 (0.3)	20.8 (0.5)
Max. ionagar + autoclaved exudate	21.8 (0.5)	24.5 (0.5)	22.1 (0.6)

Exudate dose - 1mg. dry weight of root (Clone C) per well.

The stimulation of growth was similar to that obtained with root sectors on maximum ionagar, and was uneffected by the exudation medium ($P<0.01$). The results also indicate that the factor stimulating growth is heat stable.

These results were confirmed in an experiment using the auxanographic technique (Fig. 25). The incubation period was 12 days.

In all subsequent experiments exudates were collected in distilled water for a period of 7 days.

The results of two experiments in which a range of exudate concentrations was used are given in Figs. 26 and 27. The incubation periods were 16 days. An exudate dose as low as 0·1mg. dry weight of root stimulated the growth of the fungus ($P<0·05$). The heat stable nature of the factor stimulating growth was confirmed, and there was a slight though insignificant ($P>0·05$) increase in stimulatory activity of the exudate after autoclaving.

As a further check on the inabilityof killed roots to stimulate mycelial growth, exudate was collected from killed roots and was tested over a range of concentrations (Fig. 28). The incubation period was 16 days. There was no significant effect on the growth of the fungus.

Further experiments were conducted with extracts and homogenates of excised tomato roots. The results are given in Figs. 29, 30 and 31. The incubation periods were 16 days. No,significant stimulation of growth occurred with any of the preparations, but higher concentrations of the homogenates, particularly the non-extracted form, significantly inhibited growth ($P<0·05$).

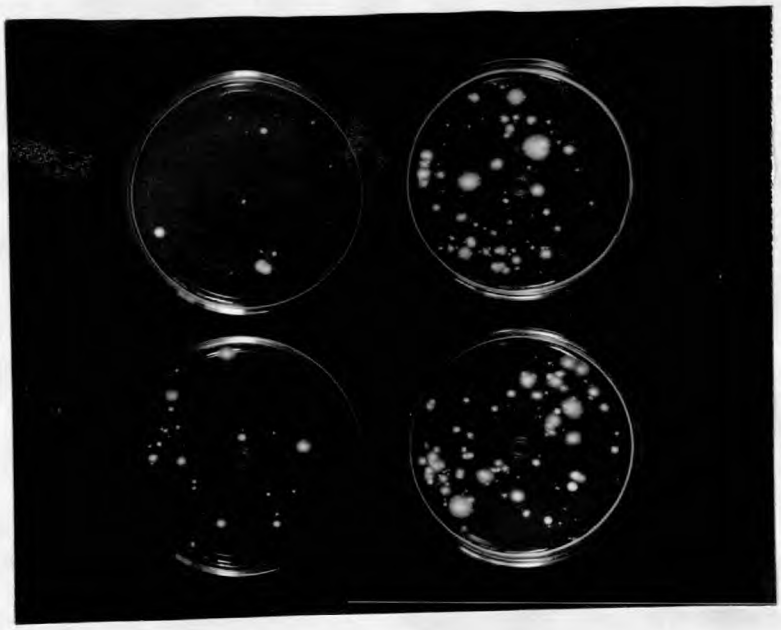


Fig. 25. Auxanographic technique. Growth of Boletus variegatus - controls (left) and with exudate equivalent to 1mg. dry weight of excised tomato root, Clone C (right).

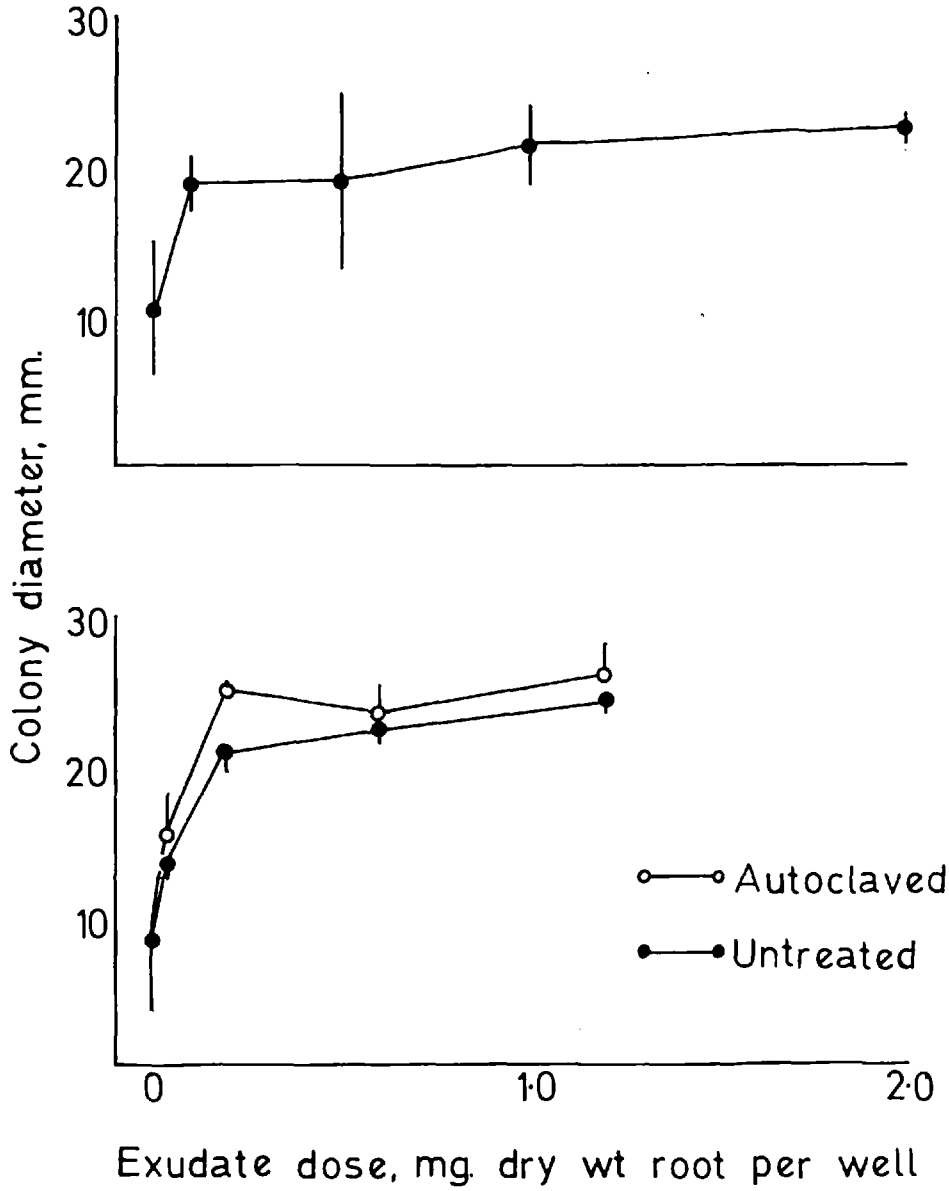


Fig. 26. Effect of excised tomato root exudate (Clone C) on the growth of Boletus variegatus.

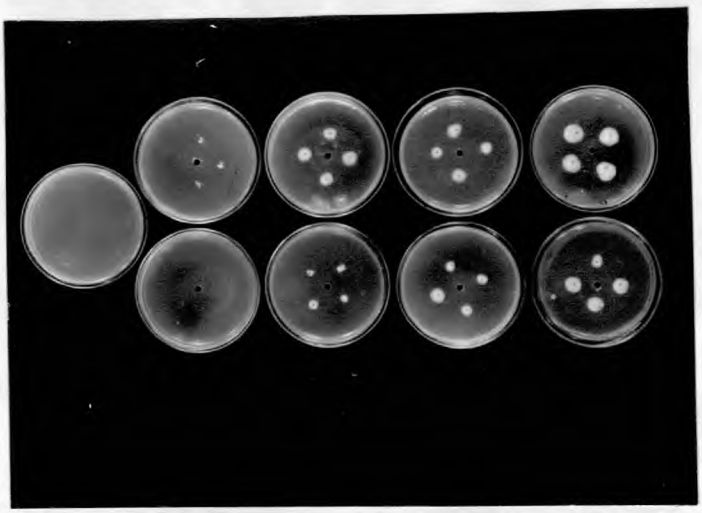


Fig. 27. Effect of excised tomato root exudate (Clone C) on the growth of Boletus variegatus. Exudate dose from left to right - 0, 0·04, 0·2, 0·6 and 1·2mg. dry weight of excised tomato root per well. Autoclaved exudate (top row) and micropore-filtered exudate (bottom row).

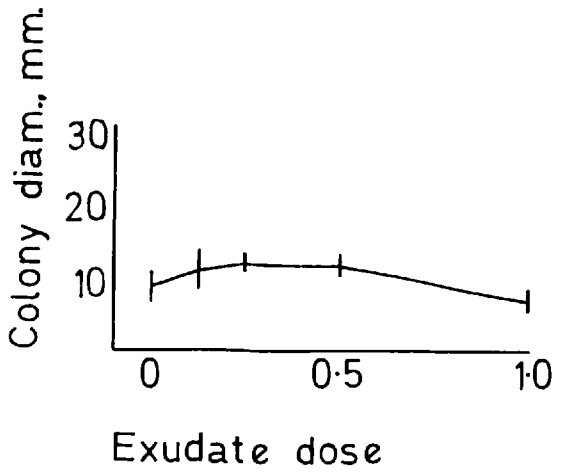


Fig. 28. Effect of killed excised tomato root exudate (Clone X) on the growth of Boletus variegatus

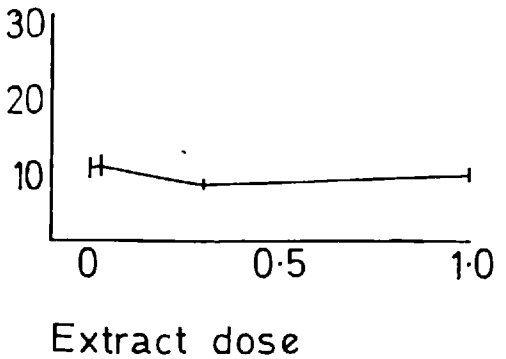


Fig. 29. Effect of excised tomato root extract (Clone X) on the growth of Boletus variegatus.

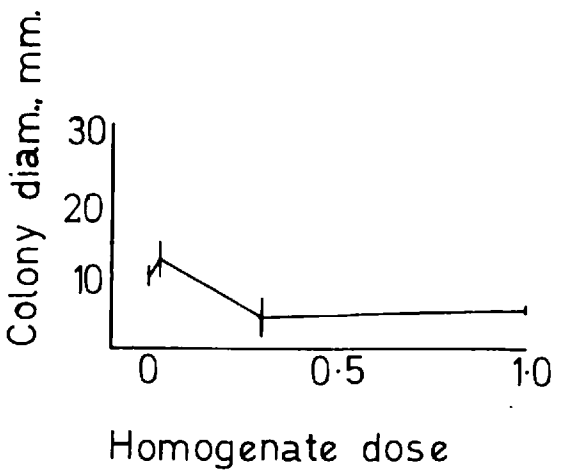


Fig. 30. Effect of excised tomato root homogenate (Clone X) on the growth of Boletus variegatus.

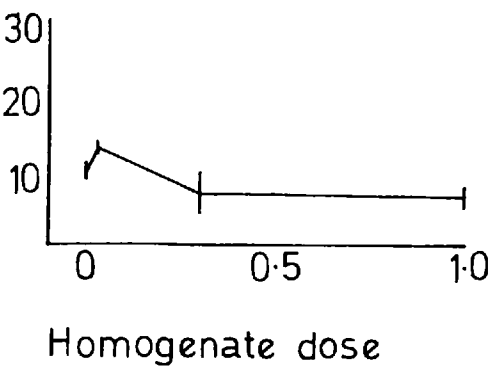


Fig. 31. Effect of excised tomato root homogenate, previously extracted (Clone X) on the growth of Boletus variegatus.

Finally to check whether the factor stimulating mycelial growth is inorganic, water-soluble ash of excised tomato root was tested over a range of concentrations (Fig. 32). Agar inocula were used. There was no effect on the growth of the fungus.

Chromatography of root exudates.

As one possible approach to the problem of identifying the growth stimulatory factor in root exudates, chromatograms were run to test for the presence of certain groups of substances known to be exuded by roots.

Amino acids and closely related compounds were identified, in two cases tentitatively, by comparing the spots with markers. The substances found are shown in Figs. 33 and 34, and the approximate concentrations are given below.

TABLE 23. Amino acids in excised tomato root exudates (Clone C).

Amino acid	Approximate conc. - micrograms per 10mg. dry weight of root.
Glutamine	50
Asparagine	20
Leucine	5
Glutamic acid	5
Phenylalanine	5

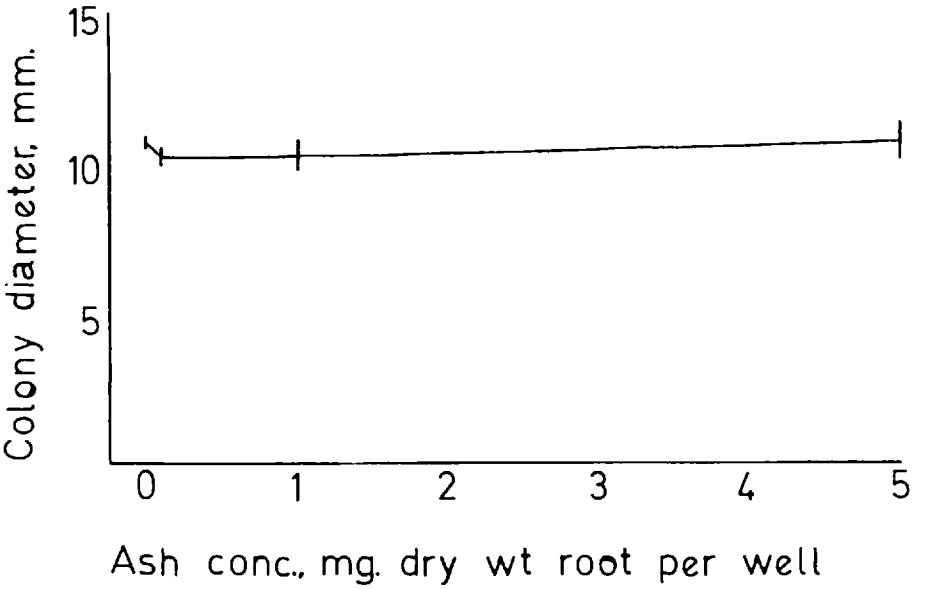


Fig. 32. Effect of excised tomato root ash (Clone C) on the growth of Boletus variegatus.

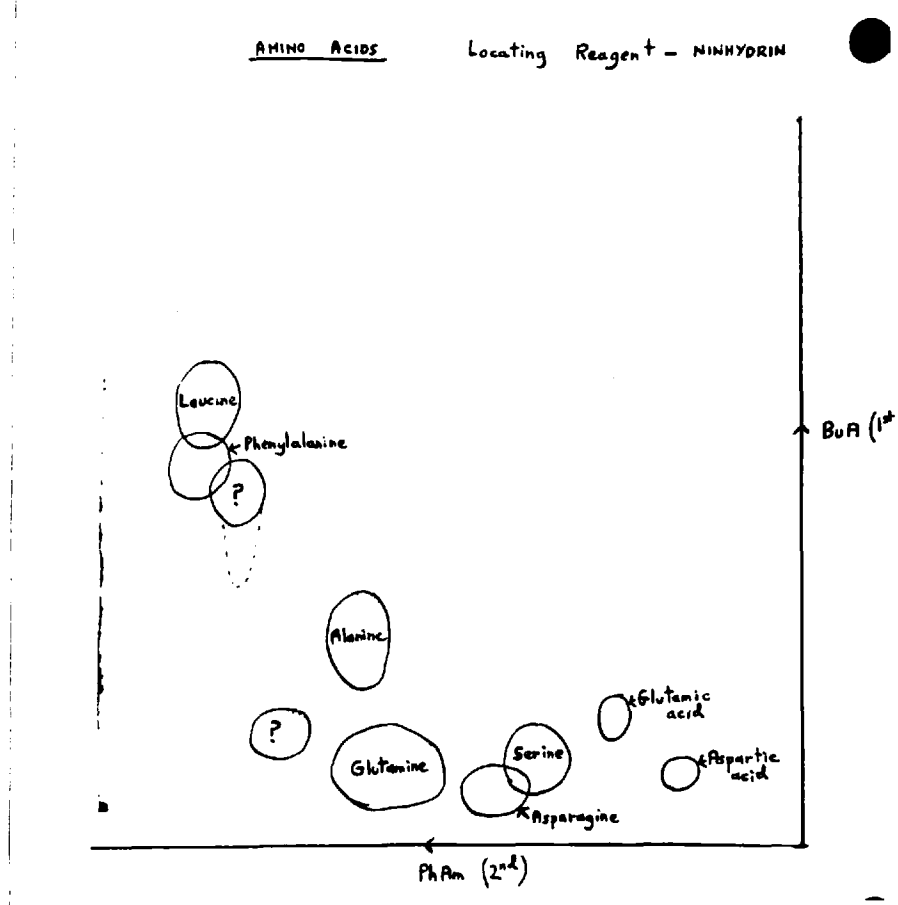


Fig. 33. Amino acids in excised tomato root exudate (Clone C).

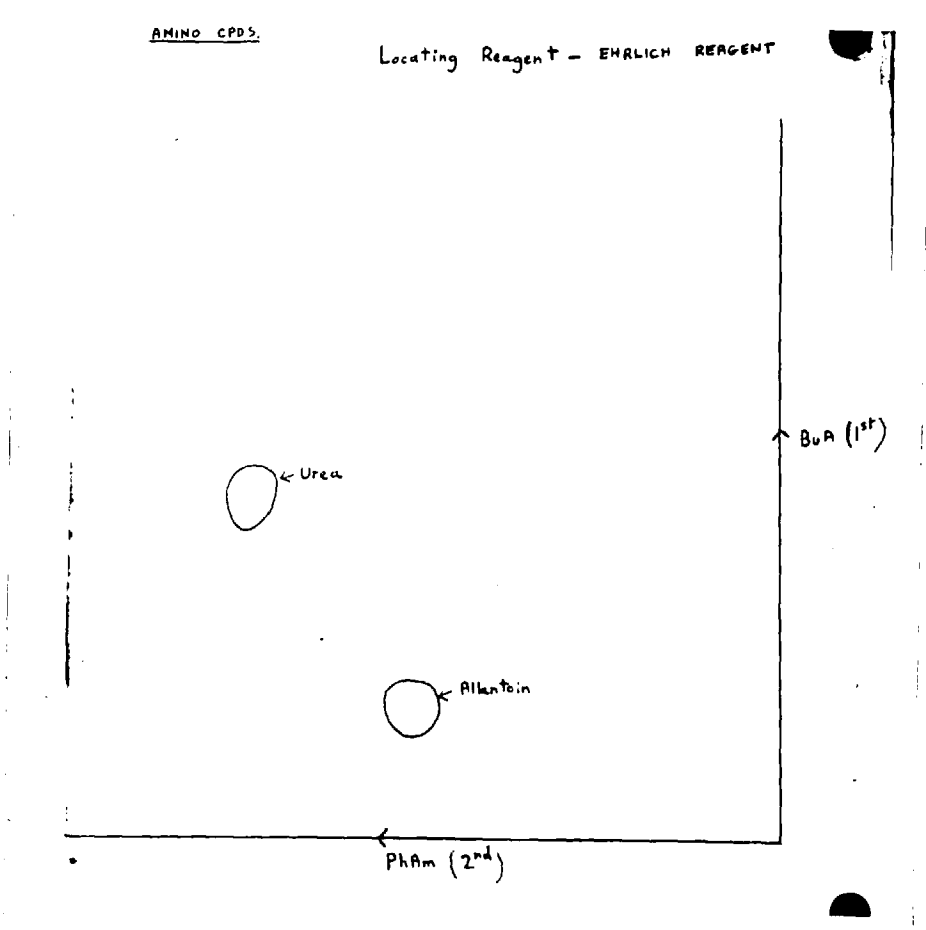


Fig. 34. Other amino compounds in excised tomato root exudate (Clone C).

Table 23 continued:

Methionine?	5
Alanine	5
Serine	5
Aspartic acid	1
Methionine sulphone?	1
Urea	not estimated
Allantoin	" "

All of the spots were located at room temperature, and no additional spots appeared on heating the dipped chromatogram at 105°C. for 3 minutes.

A number of sugars were found and the results are summarized below.

TABLE 24. Sugars in excised tomato root exudates (Clone C).

Sugar	Locating reagents	Colour	RG
Glucose	Aniline-diphenylamine	blue-grey	69 (RF)
	Anisidine HCl	brown	68 (RF)
Fructose	Anisidine HCl	yellow	108
	Naphthoresorcinol	red	107
Sucrose	Aniline-diphenylamine	brown	62
Unknown	Aniline-diphenylamine	brown	237
	Anisidine HCl	yellow	237

Table 24 continued:

Unknown	Aniline-diphenylamine	blue	25-35
	Anisidine HCl	brown	25-35
Unknown	Elson-Morgan	-	47
Unknown	Elson-Morgan	-	123
Unknown	Anisidine HCl	pink	131

Neither of the Elson-Morgan-positive spots corresponds to a commonly occurring amino-sugar. Because the region at RG 47 is also ninhydrin-positive it is possible that the reaction here is due to an amino acid.

A comparison of the sugars produced during the first day and the subsequent 6 days of exudation gave the following results.

TABLE 25. Sugars in excised tomato root exudates (Clone C).

Sugar	1st days exudate	2nd to 6th days exudate
Glucose	present	absent
Fructose	present	trace
Sucrose	present	absent

Virtually all of the three main sugars is lost from

the roots during the first 24 hours of exudation.

Chromatograms were run to check for phenolic compounds, but no attempt was made to identify the spots. The results are summarized below.

TABLE 26. Phenolic compounds in excised tomato root exudates (Clone C).

RF	Locating reagent	Reaction
33	U.V. light	blue fluorescence
	$FeCl_3$-K_3FeCN_6	positive
	Diazotized sulphanilic acid	red
97	U.V. light	blue fluorescence
	$FeCl_3$-K_3FeCN_6	positive
	Diazotized sulphanilic acid	yellow-brown

Finally inorganic ions of the alkaline earth group were tested for. Oxine treatment, followed by exposure to ammonia and then viewing in U.V. light revealed high concentrations of calcium and magnesium. It has already been shown that only Boletus elegans of the primary isolates is stimulated by calcium.

Because of the large amounts of glutamine and asparagine present in the exudates, the effect of these

compounds on growth was tested for. The results are given in Fig. 35. Asparagine stimulated growth slightly, but not to an extent comparable with roots or root exudates.

Purine derivatives and growth of the fungi.

It has been reported that nicotinamide adenine dinucleotide (NAD) can replace the growth stimulatory factor exuded from excised roots (Nilsson, 1960). The effect of NAD on growth was therefore examined, and the results of experiments are given in Figs. 36 and 37. Sigma NAD stimulated growth at low concentrations, but the B.D.H. NAD had no effect on growth. These results were confirmed in a f further experiment, the results of which are given below.

TABLE 27. Effect of NAD on the growth of Boletus variegatus.

Dry weight in mg.

	NAD source:-	
Medium	Sigma	B.D.H.
Maximum	8·4 (0·3)	
Maximum + NAD	11·9 (0·3)	8·5 (0·1)

NAD dose - 0·1mg. per flask.

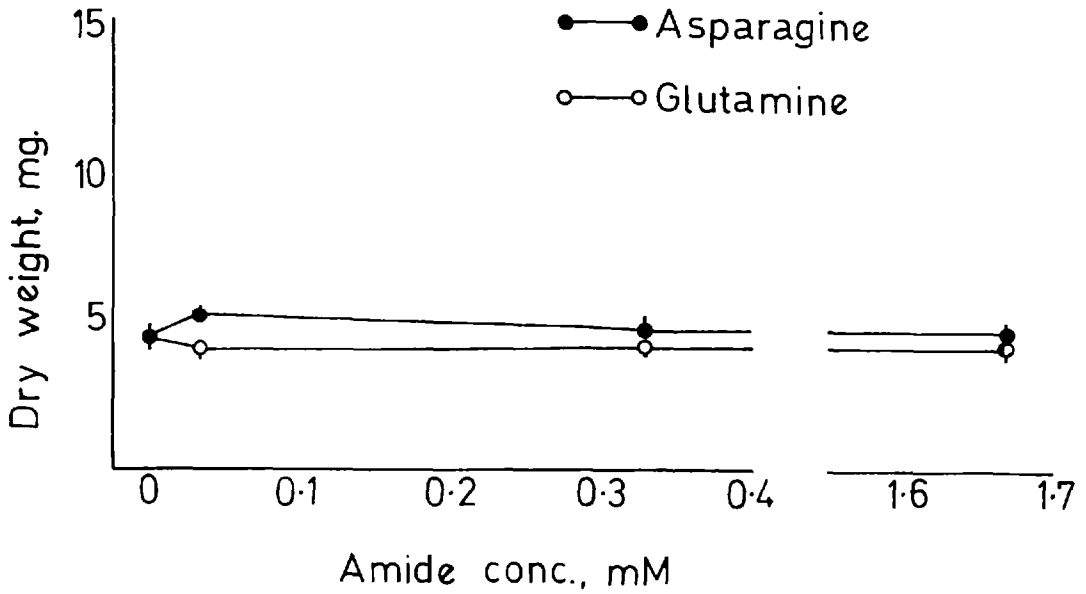


Fig. 35. Effect of asparagine and glutamine on the growth of Boletus variegatus.

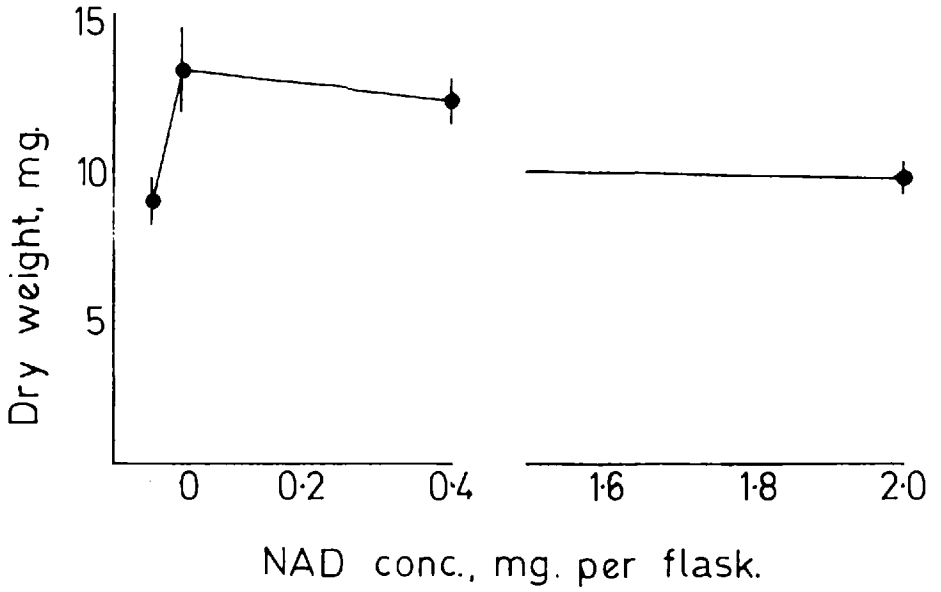


Fig. 36. Effect of NAD (Sigma) on the growth of Boletus variegatus.

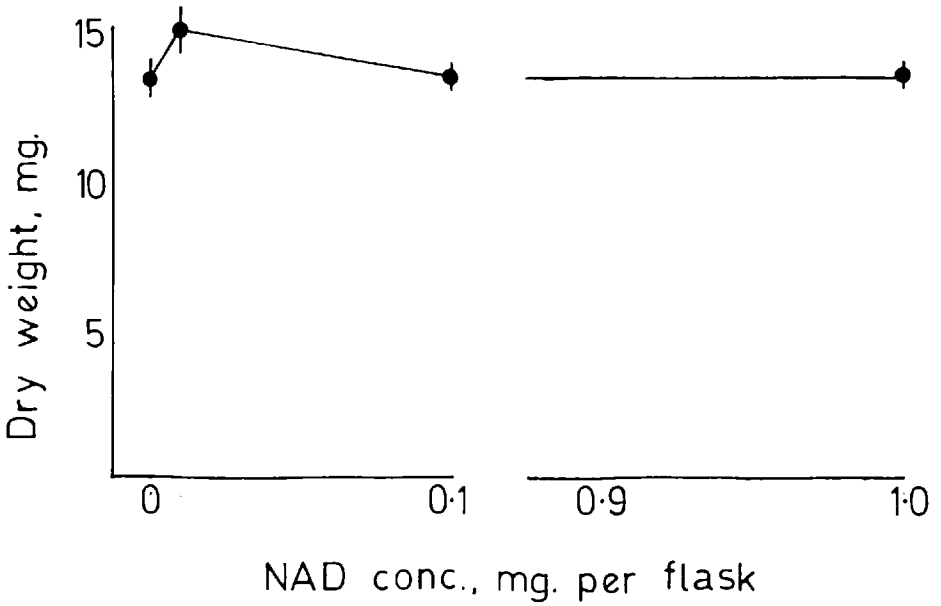


Fig. 37. Effect of NAD (B.D.H.) on the growth of Boletus variegatus.

Growth was again stimulated by Sigma NAD ($P<0.01$), but not by L.D.H. NAD.

One possible explanation for these results is the presence of a contaminant growth stimulatory substance in Sigma NAD, possibly a purine derivative. Kinetin is such a substance, which is known to be active at very low concentrations in higher plants. It's effect on the growth of the fungi was therefore examined. The results of two experiments are given in Figs. 38 and 39. The growth of Boletus elegans and Boletus variegatus was stimulated, but not to an extent comparable with roots or root exudates.

These results, although rather inconclusive, indicate that purine compounds in general may stimulate the growth of the fungi slightly, as Melin (1959) found for certain strains of Boletus variegatus. Therefore the effects of equivalent amounts of adenine and kinetin on growth were compared. The results are given in Fig. 40. The slight stimulation of growth obtained with kinetin can be accounted for by the adenine part of the molecule. Higher concentrations of adenine, as used by Melin, were still slightly stimulatory, i.e. up to 0·2mM.

Basidiospore germination.

The experiments on basidiospore germination are divided into two series: those done with collections of

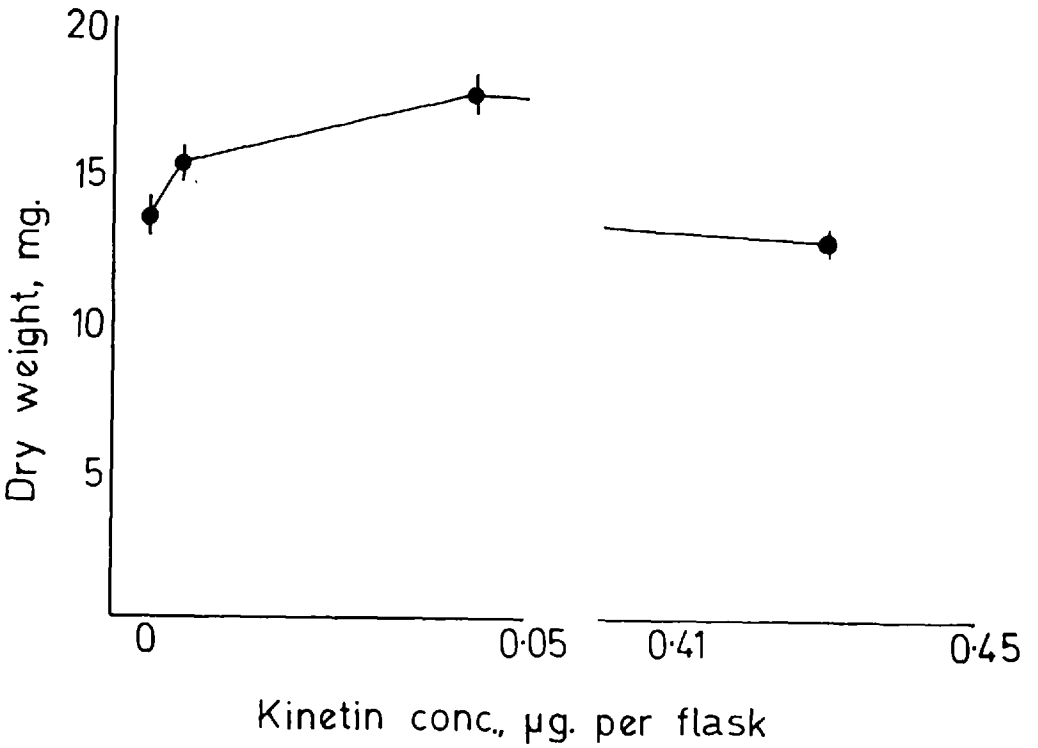


Fig. 38. Effect of kinetin on the growth of Boletus variegatus.

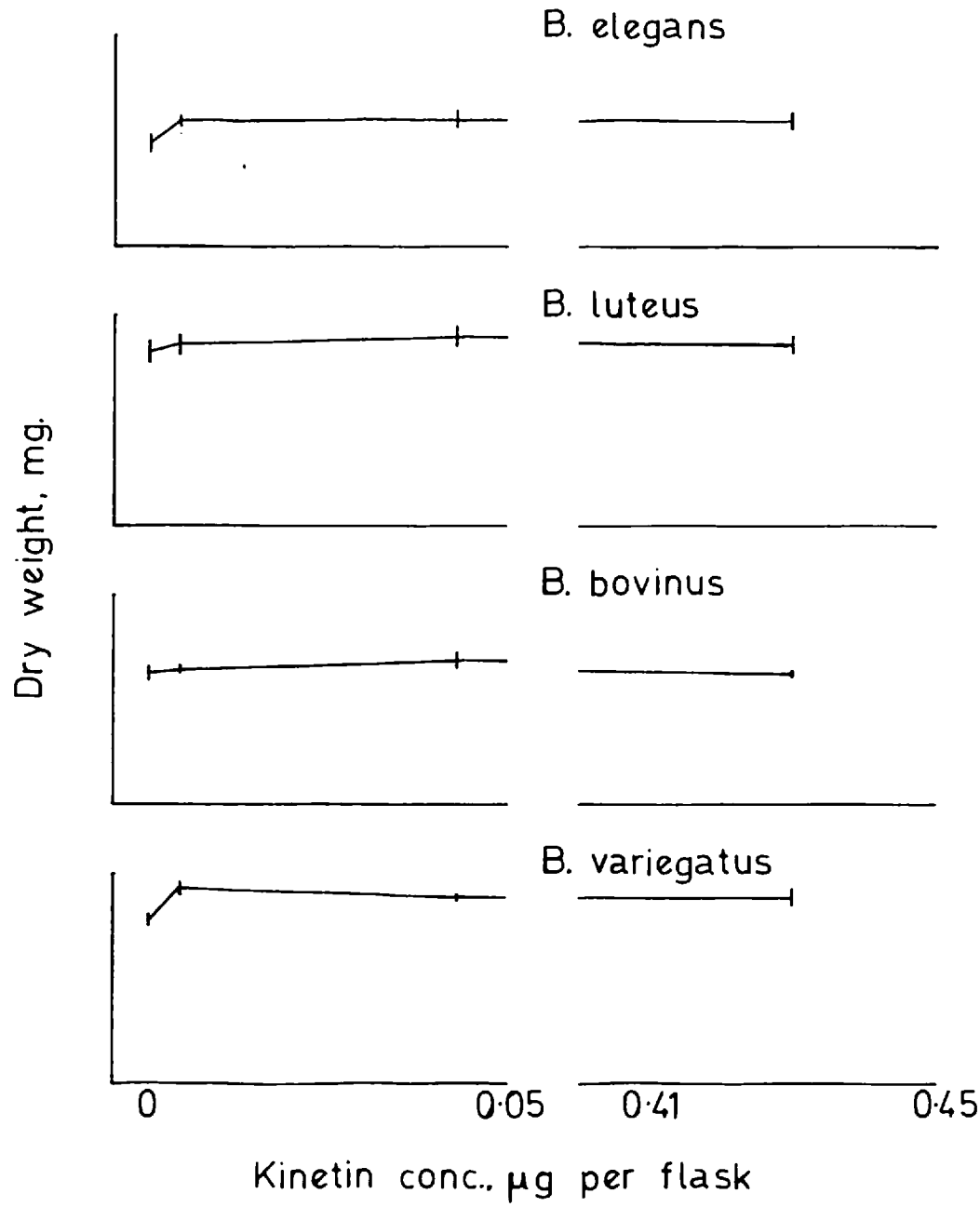


Fig. 39. Effect of kinetin on the growth of the primary isolates.

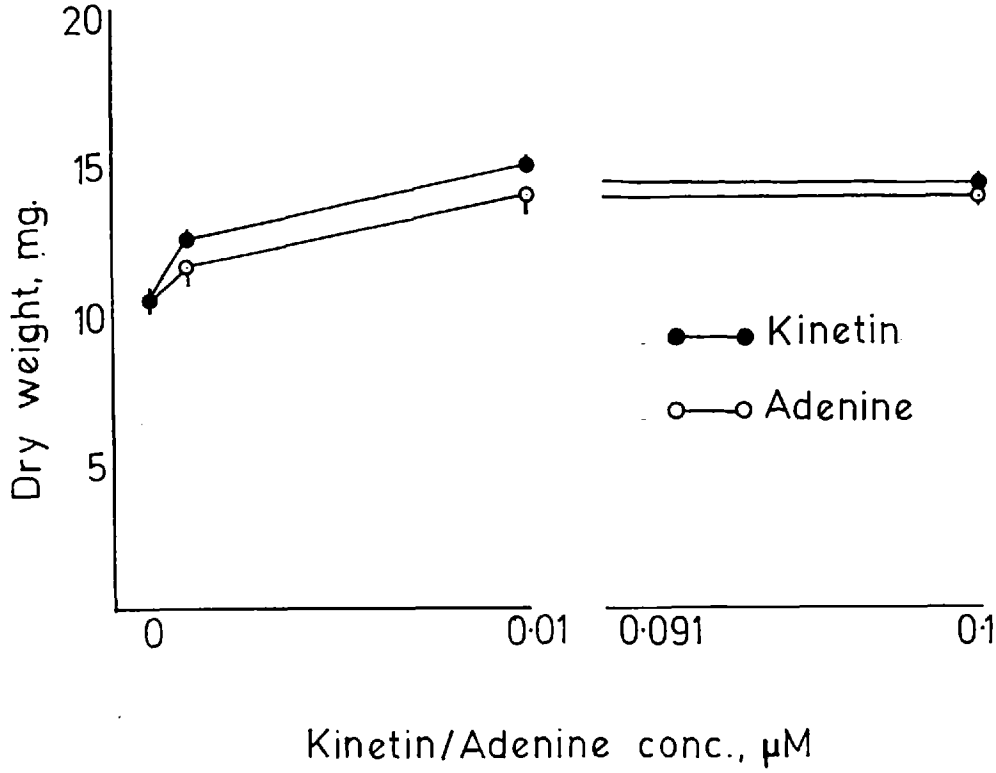


Fig. 40. Effect of equivale nt amounts of kinetin and adenine on the growth of Boletus variegatus.

Boletus luteus spores (1a-b) aquired in October, 1962, and those done with collections of spores of various Boletus species aquired in September and October, 1963.

In an initial experiment the aim was to obtain some level of germination, and therefore various media were tried in the presence and absence of the yeast Rhodotorula rubra.

TABLE 23. Germination of basidiospores.

Spore collection and concentration	Treatment	% germination on agar:- Malt	Hagem	Complete
B. luteus 1a (1962) (33×10^4 spores/ml.)	nil	0	0	0
	+ yeast	0·04	0	0
B. luteus 1b (1962) (22×10^4 spores/ml.)	nil	0	0	0
	+ yeast	0·05	0	0

There was a low level of germination on malt extract agar, but none on Hagem or Complete agar. The yeast grew very poorly on the latter.

A further experiment was conducted to test the effect of roots of tomato, Pinus sylvestris and Larix decidua seedlings on spore germination.

TABLE 29. Effect of roots on basidiospore germination.

Spore collection - Boletus luteus 1b (1962); concentration 36×10^4 spores/ml.

% germination with:-				
Control	Yeast	Tomato	Pine	Larch
0·0005	0·04	0·06	0·09	0·08

Roots stimulated germination to about the same extent as the yeast, but direct comparison is not really meaningful. Fig. 41 shows spores of Boletus luteus germinated in the presence of yeast and roots, and Fig. 42 a single germinated spore.

Finally in this first series of experiments the effect of root exudate on germination was examined.

TABLE 30. Effect of root exudate on basidiospore germination.

Spore collection - Boletus luteus 1b (1962); concentration 45×10^4 spores/ml.

% germination with:-				
Control	Excised tomato root	Exudate	Aut. exudate	Yeast
0·005	0·03	0·03	0·02	0·05

Exudate dose - 1mg. dry weight of root (Clone C) per well

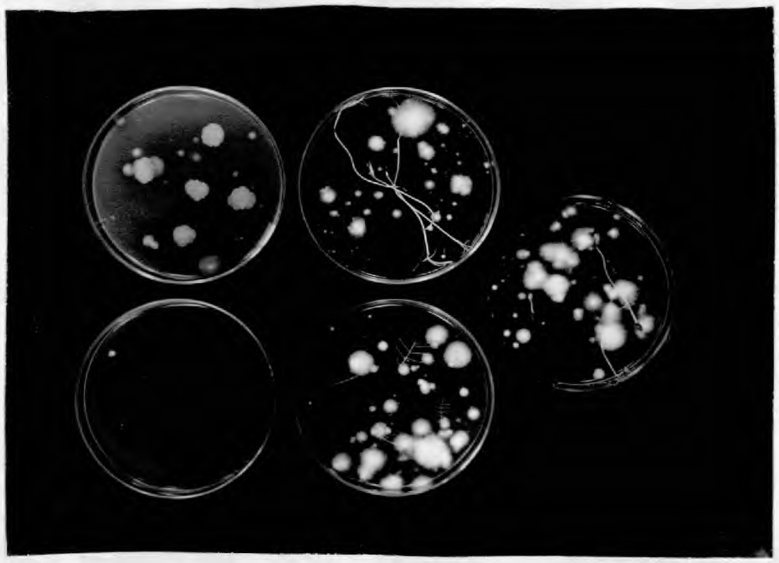


Fig. 41. Germination of basidiospores of Boletus luteus - control (bottom left), with yeast (top left), with excised tomato root sectors, Clone X (bottom centre), with pine seedling roots (top centre) and with larch seedling roots (right).

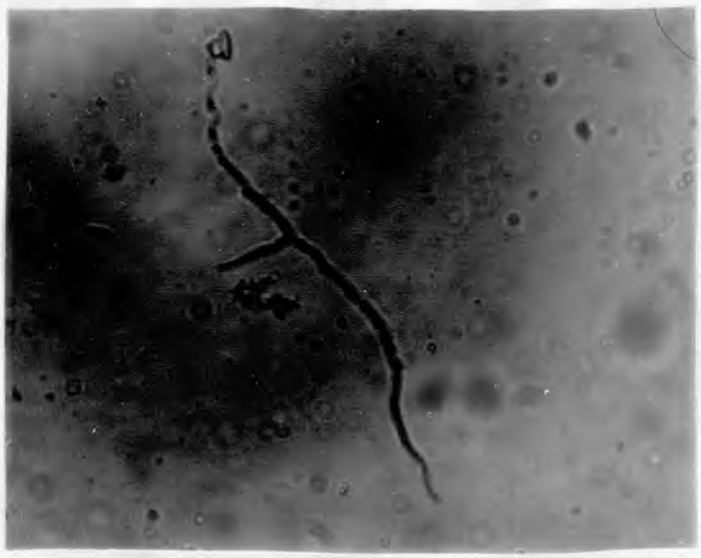


Fig. 42. Germinated spore of Boletus luteus.

Exudate of excised tomato roots stimulated spore germination, and the stimulatory factor is heat stable.

It should be noted that during the course of these experiments the spores aged from one to 120 to 190 days old, age being taken from the day of collection. During this time the percentage germination of the controls increased from zero to 0·0005 to 0·005.

In the second series of experiments a number of spore collections of five Boletus species were examined. First the effect of Rhodotorula rubra on germination was examined.

TABLE 31. Germination of basidiospores.

Spore collection (all 1963)		Age (days)	Concentration (x 10^{-4} spores/ml.)	% germination with yeast
B. elegans	1a,b,c,d	45	65,116,106,55	0
" "	2b,c,e	13	77,42,119	0
" "	2a	13	196	0·005
" "	2d	13	184	0·06
" "	2g	13	21	0·05
" "	3a,b,c,d	6	288,190,80,73	0
B. scaber	1a,b,c	13	50,60,215	0
B. luteus	2a	38	47	0·09
" "	2b	38	94	0·006
" "	2c	38	47	0

Table 31 continued:

B. luteus	2d	38	106	0·01
" "	3a	6	61	0
" "	3b	6	25	0·07
" "	3c	6	36	0·12
" "	3d	6	170	0·06
B. bovinus	1a	38	16	0·06
" "	1b	38	36	0·04
" "	1c	38	14	0·08
" "	1d	38	10	0
" "	2a	43	45	0·009
B. variegatus	1a	45	85	0·02

The level of germination was always very low, and only in one case did it exceed 0·1 per cent. No germination occurred at all in the absence of the yeast. Some collections failed to germinate even in the presence of the yeast, notably Boletus elegans collections 1a-d and 3a-d and Boletus scaber collections 1a-c.

The effect of roots on the germination of basidiospores of some of these collections was next tested.

TABLE 32. Effect of roots on basidiospore germination.

Spore collection (all 1963)	Age (days)	Concentration ($\times 10^{-4}$ spores/ml.)
B. elegans 2d	76	235
" " 2g	76	90
B. scaber 1b	76	32
" " 1c	76	83
B. luteus 2a	101	170
" " 3c	69	57
B. bovinus 1a	101	93
" " 1b	101	112
B. variegatus 1a	108	225

Spore collection	% germination with:- Control	Yeast	Tomato	Pine	Larch
B. elegans 2d	0	0·04	0·05	0·04	0·04
" " 2g	0	0·03	0·03	0·04	0·04
B. scaber 1b	0	0	0	0	0
" " 1c	0	0	0	0	0
B. luteus 2a	0·001	0·10	0·10	0·12	0·12
" " 3c	0·002	0·18	0·18	0·19	0·16
B. bovinus 1a	0	0·03	0·05	0·01	0·03
" " 1b	0·002	0·08	0·06	0·08	0·06
B. variegatus 1a	0	0·02	0·04	0·04	0·03

Roots stimulated the germination of spores of all species except Boletus scaber. Roots of Pinus sylvestris and Larix decidua seedlings were as effective as excised tomato roots (Clone N).

An experiment was carried out to test the effect of Rhodotorula rubra culture filtrate on basidiospore germination.

TABLE 33. Effect of yeast culture filtrate on germination.

Spore collection	Age (days)	($\times 10^{-4}$ spores/ml.)	% germination with:- Control	Yeast	C.f.
B. elegans 2d	146	118	0	0·006	0
B. scaber 1b	146	18	0	0	0
B. luteus 3c	139	37	0·003	0·14	0·03
B. bovinus 1b	171	33	0·001	0·06	0
B. variegatus 1a	178	50	0·001	0·02	0

Yeast culture filtrate stimulated the germination of spores of Boletus luteus, collection 3c, but the level of germination was much lower than with the yeast itself.

This result was confirmed in a further experiment in which a range of concentrations of culture filtrate was tested (Fig. 43). Again the culture filtrate induced a low

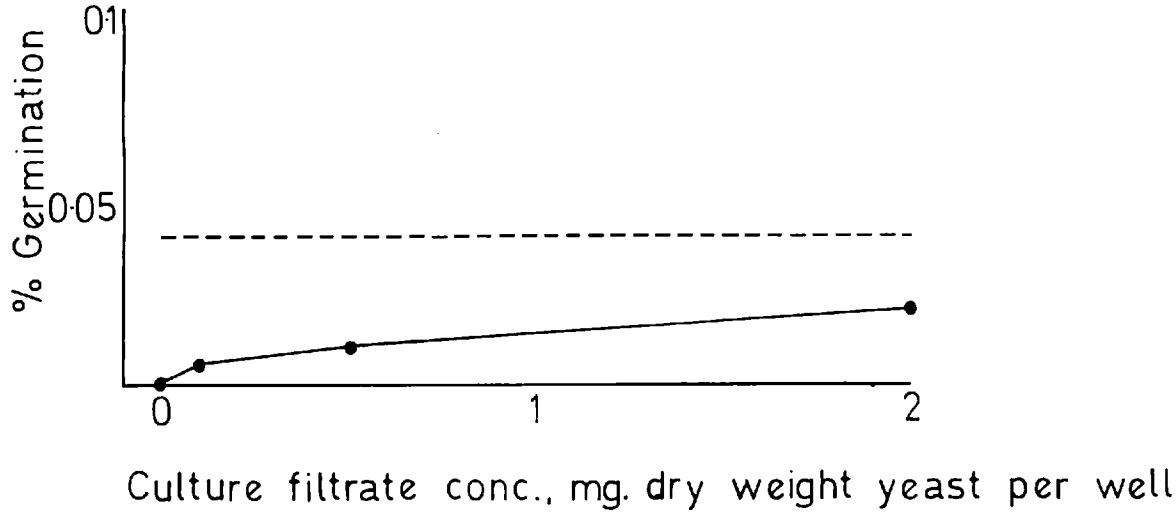


Fig. 43. Effect of culture filtrate of Rhodotorula rubra on the germination of basidiospores of Boletus luteus (collection 3c). The broken line shows the level of germination in the presence of the yeast itself.

level of germination.

Extracts of soils bearing mycorrhizas were tested to see whether such soils contain substances capable of stimulating basidiospore germination.

TABLE 34. Effect of soil extracts on basidiospore germination.

Spore collection	Age (days)	Concentration (x 10^{-4} spores/ml.)
B. elegans 2d	230	135
B. luteus 2d	255	30
" " 3c	223	24
B. bovinus 1a	255	21
B. variegatus 1a	262	41

Spore collection	% germination with:- Control	Yeast	Extract A	Extract B
B. elegans 2d	0	0	0	0
B. luteus 2d	0•01	0•03	0•01	0•01
" " 3c	0	0	0	0
B. bovinus 1a	0	0•1	0	0
B. variegatus 1a	0	0•0%	0	0

Extract A - from soil bearing B. elegans

Extract B - from soil bearing B. bovinus

Soil extracts did not stimulate the germination of basidiospores. Two of the spore collections showed complete loss of viability, and in one case, Boletus luteus 2d, the level of germination in the controls was very high.

Finally in view of the fact that roots stimulate both mycelial growth and basidiospore germination, it was decided to test the effect of Rhodotorula rubra on mycelial growth. Plates of malt extract agar were co-inoculated with the yeast and the Boletus species (agar inoculum). The results are given in Fig. 44. The yeast did not stimulate growth of the fungi.

Interactions between Boletus species and other fungi.

Interactions between Boletus species and other fungi, with which they might compete in vitro, were considered to be of some interest. Experiments were therefore carried out with the primary Boletus isolates to test for any interactions, first between various Boletus species, secondly between Boletus species and non-mycorrhizal Hymenomycetes and thirdly between Boletus species and other soil fungi.

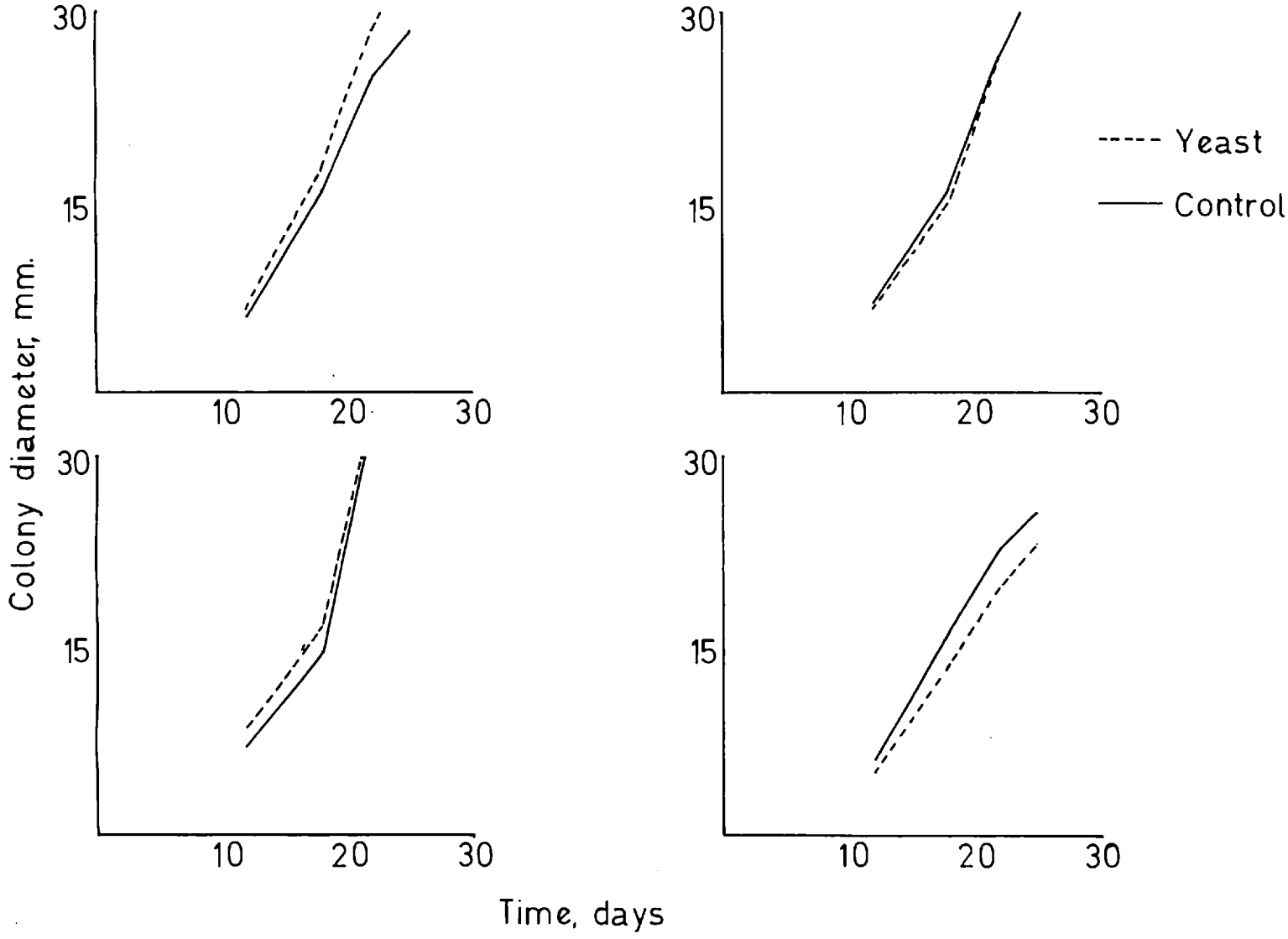


Fig. 44. Effect of Rhodotorula rubra on the growth of the primary isolates.

TABLE 35. Interactions between Boletus species.

	Colony diameter in mm. at 15 days			
Inoculated x	B. elegans	B. luteus	B. bovinus	B. variegatus
B. elegans	23·0 (1·2)	27·8 (0·6)	27·5 (0·8)	24·8 (1·1)
B. luteus	23·0 (0·9)	32·6 (1·0)	26·5 (0·6)	20·8 (1·1)
B. bovinus	21·8 (0·7)	33·8 (1·7)	25·4 (0·8)	22·3 (0·5)
B. variegatus	23·5 (1·4)	33·0 (1·4)	25·0 (1·3)	24·4 (1·0)

At this stage of development, 15 days, the colony edges were still 5 to 12 mm. apart. By 20 days Boletus elegans had formed a white, crusty looking growth at the points of intersection with the mycelia of the other species (Fig. 45). Marked inhibition zones were visible between Boletus elegans and Boletus bovinus, and between Boletus bovinus and Boletus variegatus.

TABLE 36. Interactions between Boletus species and non-mycorrhizal Hymenomycetes.

	Colony diameter in mm. at 8 days			
Inoculated x	B. elegans	B. luteus	B. bovinus	B. variegatus
Control	10·0 (0·6)	16·5 (1·2)	12·0 (0·4)	12·5 (1·0)
M. fasciculare	11·3 (0·5)	18·3 (1·2)	12·8 (0·7)	13·3 (1·0)
M. alliaceus	6·8 (0·4)	7·3 (0·8)	5·3 (0·2)	4·8 (0·3)

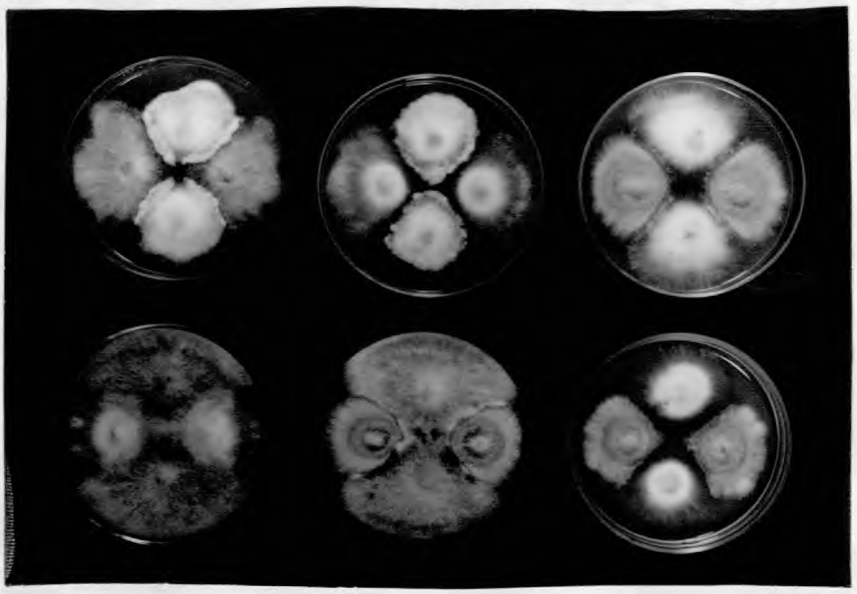


Fig. 45. Interactions - B. elegans x B. luteus (top left), B. elegans x B. bovinus (top centre), B. elegans x B. variegatus (top right), B. luteus x B. bovinus (bottom left), B. luteus x B. variegatus (bottom centre) and B. bovinus x B. variegatus (bottom right). The top and bottom colonies are given first and the lateral ones second.

Marasmius alliaceus strongly inhibited the growth of the Boletus species. After 3 weeks inhibition zones were formed with Boletus elegans and Boletus luteus, and later, after 4 to 5 weeks, Marasmius alliaceus overgrew the Boletus species. The growth of Marasmius alliaceus was not influenced by the Boletus species.

Hypholoma fasciculare formed ill-defined inhibition zones after 2 weeks with Boletus luteus and Boletus variegatus, but there was no inhibition of growth of the Boletus species before this occurred. Hypholoma fasciculare was not effected by the Boletus species and it eventually overgrew them.

Because the growth rate of Clitocybe infundibuliformis was very slow, this fungus was inoculated onto the plates 12 days before the Boletus species.

TABLE 37. Interactions between Boletus species and Clitocybe infundibuliformis.

	Colony diameter in mm. at 15 days			
Inoculated x	B. elegans	L. luteus	B. bovinus	B. variegatus
Control	24·3 (0·9)	29·0 (2·1)	28·5 (1·4)	25·6 (1·7)
C. infundibuliformis	21·0 (1·2)	6·3 (0·8)	13·5 (0·9)	12·8 (0·9)

All of the Boletus isolates were markedly inhibited by Clitocybe infundibuliformis, except for Boletus elegans. After 3 weeks Boletus elegans ceased growth at about 4mm. from the mycelium of Clitocybe infundibuliformis, whereas the other Boletus species showed very little growth beyond that established at 15 days. The growth of Clitocybe infundibuliformis was slightly retarded in the later stages,,but because of the very irregular outline of the colonies growth could not be satisfactorally assessed.

Trametes versicolor grew across the plates very rapidly and overgrew the Boletus species within 4 days, with no effect on growth rates or formation of inhibition zones.

TABLE 38. Interactions between Boletus species and non-mycorrhizal Hymenomycetes.

	Colony diameter in mm. at 15 days			
Inoculated x	B. elegans	B. luteus	B. bovinus	B. variegatus
Control	22•4 (1•4)	25•2 (1•3)	30•0 (2•8)	23•4 (1•7)
A. mellea	22•3 (1•1)	26•1 (0•7)	27•3 (2•1)	22•9 (1•6)
P. betulinus	20•9 (0•9)	28•3 (1•5)	28•2 (2•4)	24•1 (2•3)
Fomes sp.	22•4 (1•0)	28•3 (2•3)	29•4 (2•0)	25•0 (2•6)

The Boletus species were not inhibited by the non-

mycorrhizal Hymenomycetes. Piptoporus betulinus and the Fomes species were both slightly inhibited in the later stages.

For the third experiment fungi were isolated from the soils previously used for the experiment on basidiospore germination. Two commonly occurring fungi were selected, a Trichoderma species and a Penicillium species. In addition a laboratory contaminant, a second Penicillium species, which had been observed to inhibit the growth of the Boletus species, was included. The Boletus species were inoculated onto the plates 7 days before the soil fungi.

TABLE 39. Interactions between Boletus species and other soil fungi.

	Colony diameter in mm. at 11 days			
Inoculated x	B. elegans	B. luteus	B. bovinus	B. variegatus
Control	19·6 (1·3)	25·3 (1·5)	22·1 (1·3)	20·9 (1·4)
Trichoderma	14·3 (1·0)	21·3 (1·0)	16·8 (0·5)	15·1 (1·2)
Penicillium	16·0 (1·0)	25·8 (1·7)	17·3 (0·9)	15·6 (1·5)
Penicillium (lab. isolate)	14·5 (0·7)	19·6 (0·8)	17·1 (0·7)	16·2 (0·9)

All of the Boletus isolates were inhibited by the soil fungi except for Boletus luteus.

DISCUSSION

Vigour and infectivity of the fungi.

The technique used for isolating cultures is a standard one for fungi which do not sporulate *in vitro*, and which produce large sporophores *in vivo*. Cultures so obtained consist of dikaryotic mycelium. No attempt was made to make the cultures genetically stable by isolating from hyphal tips, but care was taken to avoid subculturing from abnormal sectors which were occasionally produced by some isolates of *Boletus elegans*.

Two media, malt extract agar and Lindeberg agar, were used initially for the maintenance of stock cultures, because previous wotkers had reported that continuous culture on a single medium resulted in a loss of vigour. However, because cultures of the primary isolates were also kept under liquid paraffin, it was later decided to use a single medium, namely modified Lindeberg agar, for maintaining normal cultures.

Cultures not maintained under oil did eventually exhibit changes in vigour. Some isolates of *Boletus elegans* and *Boletus luteus* increased in vigour, and presumably did so by a process of selection, although, as has already been stated, care was taken to avoid subculturing from abnormal sectors. How (1940) reported no change in the vigour of an

isolate of Boletus elegans over a period of 4 years.

Most isolates of Boletus bovinus and Boletus variegatus showed a decrease in vigour, possibly due to a loss of ability to synthesize, at a sufficient rate, one or more growth factors required by the fungi and not adequately supplied by the medium. Levisohn (1944), working with isolates of Boletus bovinus, observed decreases in vigour within 3 years.

The maintenance of cultures of the primary isolates under liquid paraffin ensured that material of constant vigour was available throughout the investigations.

Details of the mycelial morphology of Boletus species in culture are rather scarce in the literature. However the details wich are available (How, 1940; Levisohn, 1944, and Hodess, 1941) are in agreement with those for the morphology of the present isolates. There are three points worthy of special note. The first is the limited penetration of the agar by the hyphae, probably correlated with a general inability among Boletus isolates to grow well at low oxygen tensions. The second is the formation of mycelial strands by many of the isolates, and the third the infrequent occurence of clamp connections in some isolates, and their complete absence in others.

The large number of failures among the attempts to synthesize mycorrhizas is not easily explained. One of the

main problems is establishing a suitable initial pH of the vermiculite medium (Macskaylo, personal communication). The initial pH was adjusted in some experiments, to give a value, after autoclaving, of 5•2. This pH supports good growth of the seedlings and the fungi individually, but does not seem to favour mycorrhizal formation. It is worth noting that mycorrhizas are reported to be most frequent *in vivo* when soil nutrients are either limiting or in an unbalanced state and when the soil pH is low.

Although the vermiculite technique has been used successfully many times, it is not unusual for failures to occur (Macskaylo, personal communication). Also, failure to form mycorrhizas *in vitro* is not conclusive evidence for an inability to form mycorrhizas *in vivo*. Therefore it has to be assumed that, under the right conditions, all of the present isolates would form mycorrhizas.

Nutrition of the fungi.

The nutrition of the primary *Boletus* isolates was investigated in some detail for two reasons. First, information in the literature on the nutritional requirements of *Boletus* species is somewhat sparse and scattered and secondly, it was thought that the results of such investigations might help to clarify the problem of the effect of root exudates on the growth of *Boletus* species.

The maximum (liquid) medium and the maximum ionagar used throughout the investigations are those used by Melin et al. (1954), designed to ensure the supply of any common growth factors likely to be required by these fungi. As will be discussed more fully later, it was found that these media areslightly sub-optimal for the growth of the primary isolates. Therefore any growth stimulation of the fungi in these media, by roots or root exudates, could be due to the neutralization of toxic levels of one or more constituents of the media.

A comparison of the growth rates of the primary isolates on maximum and basic ionagars serves to illustrate the point made above. Although the growth rate for each isolate is approximately the same on the two media, there is always an extended lag phase on the maximum ionagar, about 48 hours more than on basic ionagar. The effect of excised tomato root sectors is to shorten this extended lag phase, by rather more than 48 hours for most of the Boletus isolates. Melin's growth curves for Boletus variegatus, cultured in maximum (liquid) medium in the presence of roots or root exudates, also show a foreshortening of the lag phase, while the linear rates of growth and final yields are similar to those for the controls.(Melin et al., 1954 and Melin, 1962). However there is no evidence for the existance of a longer lag phase of growth in maximum (liquid) medium compared with basic (liquid) medium in any of Melin's experiments.

The temperature optima for the primary isolates, grown on maximum ionagar, are similar to those previously reported for Boletus species (Melin, 1925). All isolates grow best at either 25°C. or just below this temperature. Boletus bovinus grows at 30°C., and Boletus luteus and Boletus variegatus grow very slowly at 7°C. Melin (1925) reported temperature optima for the growth of isolates of Boletus luteus and Boletus variegatus at 25°C., and also some growth of both species at 5°C. How (1940) found that an isolate of Boletus elegans grew best at 21°C.

The pH curves and optima for the primary isolates are close to those previously reported for Boletus species, although different media were used. The optima lie between 4•5 and 5•5 and in no case is the optimum particularly sharp as was reported for an isolate of Boletus variegatus (Melin, 1925).

The results of experiments on carbohydrate nutrition are of interest, because this aspect of the nutrition of these fungi is so important with regard to their distribution in the soil. It is generally accepted that the fungi of ectotrophic mycorrhizas are able to utilize only simple soluble sugars, and are unable to grow on most insoluble polysaccherides. Melin (1925) noted that strains of Boletus elegans and Boletus variegatus grew well only with glucose and to a lesser extent with maltose. Mannitol supported some growth of both species,

and xylose slight growth of Boletus elegans. Sodium salts of organic acids were not utilized at all. How 91940) obtained similar results with Boletus elegans. Both workers found cellulose to be useless and starch to be utilized to a limited extent only. How also noted that Boletus elegans utilized pectin.

The results for the primary isolates confirm those above, and indicate a very similar carbon nutrition for all four species. The best growth for all isolates is with glucose. The poor growth with fructose, galactose and xylose can probably be attributed to an inefficient mechanism for the conversion of these substrates to glycolytic intermediates, although a simple inability to absorb these sugars can not be discounted.

The inability to utilize cellulose substrates is undoubtedly due to a lack of extracellular cellulases in these fungi. Similarly, in the case of tho slight utilization of soluble starch, one must assume a very limited capacity to produce extracellular hydrolytic enzymes. Strictly speaking some sort of assay with cell-free culture filtrates of the fungi is preferable to simply assessing ability to grow with the polysaccherides. However great care was taken to account for any growth due to contaminant, trace amounts of utilizabe carbon and energy sources in the media.

The limited amounts of growth of some isolates

with pectin and sodium polypectate agree with the results of How for Boletus elegans. It is to be expected that fungi which penetrate host tissues intercellularly would be capable of degrading pectic substances.

The improved utilization of starch and pectin by Boletus elegans and Boletus variegatus, when supplied with a trace amount of glucose, is interesting. Norkrans (1950) reported improved utilization of cellulose by at least on e mycorrhizal species of Tricholoma, in the presence of a trace of glucose.

The preferance for an ammonium or organic form of nitrogen, already well established for many Hymenomycetes, is evident for the primary isolates. The poor growth on nitrate almost certainly reflects an inefficiency on the part of these fungi to reduce nitrate. However the importance of pH with regard to the assimilation of ammonium and nitrate ions is well known, and the pH in the present experiment was prabably not optimum for the maximum assimilation of both ions.

The stimulation of the growth of Boletus elegans by calcium needs to be treated with caution. Similar responses by various fungi have been reported by other workers, but doubts have been expressed as to whether or not the responses are directly attributable to calcium (Lindeberg, 1944). It has already been pointed out that the maximum medium is

slightly sub-optimal for growth, and therefore calcium may simply alleviate a toxicity due to some other constituent of the medium. Whatever the mechanism of the response, it is unlikely that the stimulation of mycelial growth by root exudates is due to calcium alone. Only one of the primary isolates, Boletus elegans, is stimulated and the magnitude of the response is small.

The increase in growth of the primary isolates in the presence of amino acids and vitamins is due mainly to the vitamins, but there is a significant interaction between the amino acids and the vitamins for Boletus luteus and Boletus bovinus. Norkrans (1950) reported that for Tricholoma species the amino acid and vitamin requirements were very complex and to a large extent interdependent. In many instances she found that certain amino acids were toxic at the concentrations used, even though the amino acid mixture as a whole was at optimum stimulatory concentration. The results of the present experiments in which single amino acids, or the amino acid mixture lacking single amino acids, were used, indicate that some of the acids are similarly toxic for the primary isolates. These toxicities can not be accounted for by breakdown of the amino acids to either phenolic compounds or aromatic acids during autoclaving.

The growth responce to the vitamins is mainly due to thiamin. It is apparent from the results that the inocula

were not completely thiamin-free in spite of their being leached. Melin (1954) reported very little growth of an isolate of Boletus luteus, and negligable growth of an isolate of Boletus variegatus, in the absence of thiamin.

Roots and root exudates.

The results of experiments with excised tomato roots show that the mycelial growth of the primary isolates is markedly stimulated by roots. The magnitude of the stimulation depends on the amount and type of inoculum used. With sieved mycelial suspension, where the inoculum per flask is very small and the carry-over of any essential growth factors is at a minimum, the growth stimulation is at a maximum. Conversely, with agar inocula, where the inoculum is large and relatively undamaged, and the carry-over of essential growth factors therefore substantial, the growth stimulation is much lower.

Melin (1954) showed that, for an isolate of Boletus variegatus grown in maximum (liquid) medium, the growth curve s in the presence and absence of excised tomato roots were similar in shape and height. The essential difference was a lag of approximately 3 days in the development of the control curve. Hence the stimulatory effect of roots is only on the lag phase of growth. Obviously the point in time at which stimulation of growth will be at it's greatest is when growth

is when growth is just commencing the linear phase in the control curve. Although the present experiments were not sampled at this particular point of development, the comparisons of the various degrees of stimulation are considered reasonably valid.

The results of these initial experiments also show a marked positive interaction between the maximum medium and the tomato roots. Possibly the roots are neutralizing toxic levels of some amino acids known to be present in the maximum medium, but the magnitude of the interaction is, in most cases, too great to be accounted for by this mechanism alone.

These results support Melin's hypothesis that on e or more substances, not present in the maximum medium, and synthesized at only a limiting rate by the fungi, are exuded by excised tomato roots.

The results of experiments on maximum ionagar show that excised tomato roots stimulate the growth of mycorrhizal fungi, i.e. Boletus species, but not the growth of non-mycorrhizal Hymenomycetes. There is therefore some degree of specificity involved with regard to the stimulation of the growth of fungi by tomato roots. Lundeberg (1960) has previously observed no response of an isolate of Psalliota arvensis to roots of Pinus sylvestris seedlings in culture.

The fact that excised Pinus sylvestris roots also stimulate mycelial growth is confirmed for Boletus variegatus. It is evident from this result and many others reported in the literature, that the fungi of ectotrophic mycorrhizas respond to both host and non-host roots (Melin, 1954; Melin et al., 1954, and Melin, 1962 and 1963).

Boletus variegatus responds to tomato seedling roots in the maximum medium, but not to the same degree as to excised tomato roots. A possible explanation for this is to be found in a comparison of the effects of exudates of excised Pinus sylvestris roots and Pinus sylvestris seedling roots on growth (Melin, 1963). It was found that, above the optimum concentration of exudate for the stimulation of mycelial growth, an increase in the concentration of the exudate became inhibitory, and that the concentration at which this occurred was lower for the seedling root exudate. The tomato seedlings used in the present experiment may have been supra-optimal at 12mg. dry weight of root per flask.

The results of several experiments indicate that excised roots of tomato or Pinus sylvestris, killed by immersion in boiling water for 5 seconds, have little or no ability to stimulate mycelial growth. Boletus bovinus and Boletus variegatus are slightly stimulated, and for Boletus variegatus the degree of stimulation is greater with agar inocula than with sieved mycelial suspension. Melin (1963)

has also reported growth stimulation of an isolate of Boletus variegatus with excised Pinus sylvestris roots, which had been extracted in boiling distilled water for 5 minutes. Boletus luteus and Boletus elegans are inhibited by killed roots. There is some evidence for the existence of an inhibitory factor, which is released more easily from roots with damaged plasma membranes (Melin, 1963). This could explain the results with killed roots. More simply the explanation could be that killed roots do not exude the stimulatory factor to the same extent as living roots.

The growth of Boletus variegatus is also stimulated by excised tomato root exudate. Similar responses to exudates of excised tomato and Pinus sylvestris roots have been obtained by Melin (1962 and 1963), in maximum (liquid) medium. The degree of stimulation is comparable with that obtained on m maximum ionagar with excised tomato root sectors of approximately one mg. dry weight. However the exudate is still stimulatory at a concentration equivalent to 0·1mg. dry weight of root per plate. Melin (1963) reported that excised Pinus sylvestris root exudate is stimulatory over a range of from 0·3 to 20 units per 20ml. of maximum (liquid) medium (one unit is the exudate from one mg. dry weight of root kept in distilled water at 4°C. for 6 days). Obviously the effective concentration of exudate available to the fungus in liquid medium can not be compared with that available in the well-

plates. Also it should be noted that exudate was collected at 25°C. in the present experiments. However, Melin considers that excised tomato and Pinus sylvestris root exudates are equally effective at stimulating mycelial growth (personal communication), and the present results do not contradict this.

Melin (1963) obtained increasing inhibition of mycelial growth above 20 units of excised Pinus sylvestris root exudate per flask, and has shown that rather lower concentrations of excised tomato root exudate are inhibitory (personal communication). It is apparent that inhibitory concentrations of excised tomato root exudate were not used in the present experiments.

The inability of killed roots to stimulate mycelial growth is furhter supported by the results of an experiment with exudate collected from killed roots. The inhibition of growth at a concentration equivalent to one mg. dry weight of root per plate supports Melin's hypothesis that an inhibitory factor is released in quantity from roots with damaged plasma membranes.

With regard to the effect of extracts and homogenates of excised tomato roots the situation becomes very complex and no definite conclusions can be drawn from the results. It is evident that over the same range of concentrations as used for testing exudate, extract and homogenates do not stimulate mycelial growth. At higher concentrations non-

extracted homogenate is inhibitory. Presumably the slowly-diffusible inhibitory factor, postulated by Melin, would be liberated in large amounts from homogenized roots.

It is surprising that root extract is neither stimulatory nor inhibitory over any part of the range of concentrations applied. Melin (1962) considers that the marked inhibitory effect of extracts of excised tomato and Pinus sylvestris roots, subjected to abrasives or freeze-drying prior to extraction, is due to the damaging of root cell plasma membranes and the subsequent release of inhibitory factor. It seems surprising that plasma membranes should remain intact during extraction in boiling distilled water as is suggested by the results of the present experiment.

The result of an experiment with water-soluble ash of excised tomato roots is convincing evidence that the stimulatory factor derived from these roots is not inorganic.

These experiments with roots and root exudates were conducted with three objectives in view. The first was to confirm the results of Melin and his coworkers on the existance of the stimulatory factor exuded by roots, and the second was to look at some biological and physical properties of this factor. With regard to these two aims, the results of the present experiments generally agree with those of Melin. The third objective was to develope a technique suitable for the bioassay of the stimulatory factor derived from roots, and

this will be discussed more fully later.

It is possible that the stimulatory factor is chemically detectable at biologically active concentrations. From the results of the chromatographic investigations it is evident that a wide range of organic compounds are released from excised tomato roots suspended in distilled water.

Among the ninhydrin positive compounds detected in exudate were glutamine and asparagine. The latter stimulates the growth of Boletus variegatus but only to a slight extent.

The three main sugars identified in exudate, namely glucose, fructose and sucrose, are all adequate carbon and energy sources for Boletus species, but could hardly account for the stimulation of mycelial growth brought about by roots and root exudates. The possibility remains that some unidentified "sugar" might be important.

Phenolic compounds were detected in exudate but it is unlikely that such substances would stimulate mycelial growth. The inhibitor in exudate, reported by Melin, could be phenolic.

Apart from the fact that many groups of compounds were not tested for in exudate, it is of course possible that the stimulatory factor is not chemically detectable at biologically active concentrations. Two methods for the identification of such a substance are apparent. The most satisfactory would involve separation by chromatographic and

other techniques, and detection by bioassay. An indispensible prerequisite for such a method is a satisfactory bioassay. Although such a technique was developed, because of the time taken for it's development, it was not possible to use it during the course of these investigations.

A simple alternative, but less logical, method was employed. This involved selecting potential growth stimulating substances and testing their effect on mycelial growth in maximum medium. In view of the reports in the literature on the stimulatory activity of adenine (Melin, 1959) and nicotinamide adenine dinucleotide (Nilsson, 1960), attention was concentrated on these substances.

The results of experiments with NAD from two different sources, with kinetin and with adenine suggest that slight stimulation of the growth of Boletus variegatus by most of these compounds is probably due to the adenine part of the molecule. It is evident from the results that none of these compounds replaces the stimulatory factor exuded by roots. The magnitude of the stimulation is, in all cases, too small. Contrary to Nilsson's report NAD does not replace the stimulatory factor.

Basidiospore germination.

A limited number of experiments were performed with basidiospores of several Boletus species primarily to compare

the effects of roots and root exudates on the germination of these spores and on mycelial growth.

Coculture of the basidiospores with a suitable microorganism, in this case Rhodotorula rubra, is generally necessary to obtain any germination. In cases where germination occurs in the absence of the yeast, the level of germination is always increased with the yeast present. The consistently low levels of germination observed and the long times required for germination agree with the results of othe r workers.

Roots of tomato, Pinus sylvestris and Larix decidua stimulate germination, thus confirming the results of Melin (1955 and 1962). As with the stimulation of mycelial growth there is nothing to indicate that the germination is particularly specific. Basidiospores of Boletus elegans for example, respond equally well to tomato and Pinus sylvestris roots as they do to Larix decidua roots.

Tomato root exudate stimulated the germination of the basidiospores of one Boletus luteus collection (1b), and the activator involved appears to be heat stable. These last results together with the non-specific nature of the activator, suggest that the factor(s) involved in the stimulation of mycelial growth and basidiospore germination may be similar or even identical. This view has already been put forward by Melin (1962). If this is so then Rhodotorula rubra should be

capable of stimulating mycelial growth. The results of an experiment indicate that only Boletus variegatus shows a slight but insignificant stimulatory response. It is possible that there is an inhibitor, which is more active against mycelial growth than against basidiospore germination, in Rhodotorula rubra exudate. It is not possible to state with certainty, at this stage, that mycelial growth and basidiospore germination are stimulated by the same factor(s).

Culture filtrate of Rhodotorula rubra stimulates basidiospore germination of Boletus luteus (3c), but not the germination of basidiospores of other collections, which respond to the yeast itself.

The results of an experiment with soil extracts, obtained from soils bearing mycorrhizas, show that there is no widespread occurence of a substance which stimulates basidiospore germination in such soils.

The technique employed in these experiments is open to certain objections. The criterion of germination is the production of colonies, visible to the naked eye on agar plates, and this is not simply a measure of germination but also of subsequent mycelial growth. Plates were regularly examined, under the microscope, at the end of experiments for spores with visible germ tubes. These were very rarely seen, and so it appears that colony counts reflect germination quite accurately.

Two further criticisms are possibly more serious. Spores germinating early develope into large colonies by the time the experiments are terminated, and these could give rise to underestimates of germination by obscuring late germinating spores. This problem is partly overcome by using suitable numbers of spores per plate, and also by viewing colonies from underneath. The centres of the colonies are brown on the underside and this enables colonies within larger colonies to be distinguished. The third objection arises from the fact that the mycelia of various fungi are able to stimulate basidiospore germination (N. Fries, 1941 and 1943). It is possible that some autostimulation occured in these experiments, thus giving overestimates of germination. Fortunately the germination effects were usually absolute, i.e. germination or no germination.

Interactions between Boletus species and other fungi.

Because of the findings of Rypacek (1960) on interactions between Boletus variegatus and several non-mycorrhizal Hymenomycetes, it was considered worthwhile to carry out similar experiments in order to confirm if possible Rypacek's results, and also to investigate interactions between the primary Boletus isolates themselves.

It has been established that Boletus elegans, Boletus luteus and Boletus variegatus infect Larix species,

and that Boletus luteus, Boletus bovinus and Boletus variegatus infect Pinus species. Species which infect the same host are likely to compete with each other at the root surface. The strong interactions between Boletus elegans and the other isolates are of some significance in this respect, although it is not possible to say, without further experimentation, which organism in each case is the antagonist. It is worth noting that Boletus bovinus, with which Boletus elegans interacted most strongly, does not form mycorrhizas with Larix.

Significant interactions occurred between the Boletus species and two of the non-mycorrhizal Hymenomycetes tested, namely Marasmius alliaceus and Clitocybe infundibuliformis. These two species, both of which can be termed leaf-litter saprophytes, markedly inhibited all of the Boletus isolates. None of the remaining non-mycorrhizal Hymenomycetes tested interacted in any way with the Boletus isolates. Hypholoma fasciculare and Trametes versicolor can be classified as wood-inhabiting saprophytes and the others as wood-destroying parasites. The fact that the Boletus species did not inhibit the growth of the wood-destroying parasites is contrary to Rypacel's observations. It should be noted however, that all of the wood-destroying parasites used by Rypacek occur on conifers, whereas only Armillaria mellea of the species used in the present experiments falls into this category.

Although only two soil saprophytes were tested against the Boletus isolates, because both were widespread in soils where mycorrhizas were known to occur, and both markedly inhibited the growth of the Boletus isolates, this suggests that competition in the soil from antagonistic fungi is probably intense.

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APPENDIX A

Mycelial morphology on malt extract agar.

Boletus elegans.

Colony characteristics: either fine, white, closely-compacted aerial hyphae tending to turn pale golden-brown at the centre (isolates 1, 67, 77 and 81), or fine, whitish-yellow, compact hyphae adpressed to the medium and tending to form sectors (isolates 52 and 63). Minute, clear droplets on all hyphae tending to become larger and coloured in some isolates (1 and 77). Undersurface of mycelial mat dark brown (isolates 52 and 63), brown (67 and 81), brownish-white (1) or yellowish (77). Slight zonation in isolate 67.

Hyphae: aerial hyphae 1-3(-5) microns in diameter, usually some becoming darkly stained in older parts of the mycelium (isolates 1, 63, 77 and 81). Simple and paarige branching in all isolates, although infrequent in some (52 and 81). Substrate hyphae irregular, knobbly, highly septate, compact and often deeply staining.

Mycelial strands: present in some isolates (63 and 77). Central hyphae, 3 microns diameter, surrounded by single layer of finer hyphae.

Clamp connections: never seen.

Boletus luteus.

Colony characteristics: course, white aerial hyphae tending to turn pale yellowish-brown at centre. Minute, clear droplets on all hyphae tending to enlarge and colour. Undersurface of mycelial mat dark brown (isolates 44, 74 and 79), or yellowish-brown with darker centre (isolates 19 and 83). Zonation in isolate 79.

Hyphae: aerial hyphae 1-5 microns in diameter usually some becoming darkly stained in older parts of mycelium. Simple and paarige branching in all isolates, branches often arising from clamp connections where these occur (isolates 19, 44 and 83). Substrate hyphae irregular, highly- septate, compact and frequently deep staining.

Mycelial strands: present in all isolates. Centre core of large hyphae surrounded by one to three layers of finer hyphae. Total diameter of strands up to 20 microns,

strands often branched.

Clamp connections: present in some isolates (19, 44 and 83).

Boletus bovinus.

Colony characteristics: off-white aerial hyphae, fluffy in some isolates (70 and 78), adpressed to the medium in others (42 and 73). In isolate 42 a marginal zone, 10mm. wide, only was adpressed. Minute clear droplets on all hyphae except margin of isolate 42, tending to enlarge and colour in some isolates (42 and 70). Undersurface of mycelial mat dark brown, either entirely (isolates 70 and 78) or at the centre only (isolates 42 and 73).

H Hyphae: aerial hyphae 1-9 microns in diameter, sometimes tending to bec ome darkly stained in older mycelium (isolates 70 and 78). Simple and paarige branching in all isolates, often arising from clamp connections where these occur (42, 70 and 78). Substrate hyphae highly septate, compact and colourless.

Mycelial strands: present in some isolates (70 and 78), total dia meters up to 25 microns.

Clamp connections: present in some isolates (42, 70 and 78).

Boletus variegatus.

Colony characteristics: pale brownish-white aerial hyphae, colony margins irregular in some isolates (50 and 82). Clear droplets, tending to enlarge but not colouring. Undersurface of mycelial mat grey-brown with whitish margin. Zonation in some isolates (50, 55 and 82).

Hyphae: aerial hyphae 2-7 microns in diameter, usually some becoming darkly stained in older mycelium (72 and 82). Simple and paarige branching in all isolates, sometimes arising from clamp connections where these occur (isolates 50, 55 and 82). Substrate hyphae irregular, highly septate, compact and colourless.

Mycelial strands: present in isolate 50, diameter up to 15 microns.

Clamp connections: present in some isolates (50, 55 and 82).

Mycelial morphology on ionagar.

Boletus elegans (isolate 1).

Basic ionagar: pure whit, fine aerial mycelium, adpressed to medium over marginal 5mm. Slightly yellow-brown at centre due to coloured droplets.

Maximum ionagar: as on basic ionagar, but more compact with no adpressed marginal zone.

Boletus luteus.(isolate 19).

Basic ionagar: brownish, fluffy aerial mycelium with clear droplets on hyphae. Margin of colony very irregular with distinct mycelial strands spreading over agar. Undersurface soft chocolate brown with yellowish margin, 10 to 12mm. wide.

Maximum ionagar: brownish-white aerial mycelium. Droplets on hyphae becoming larger and coloured towards the c centre of the colony. Margin of colony entire. Undersurface with yellowish marginal zonn only 6-7mm. wide.

Boletus bovinus (isolate 42).

Basic ionagar: patchy white and yellowish-brown fluffy aerial mycelium. Colony margin slightly irregular. Few clear droplets on hyphae, and mycelial strands present near the colony margin. Undersurface ver deep rich brown, with off-white margin, 12mm. wide.

Maximum ionagar: mycelium rather more compact. Strands absent, but dense hyphal patches present near colony margin. Droplets becoming larger and coloured towards the centre of the colo ny. Underfurface with marginal zone only 5mm. wide.

Boletus variegatus (isolate 50).

Basic ionagar: brown, fluffy, rather thin aerial mycelium. Welld eveloped trands present, and some small, clear droplets on the hyphae. Undersurface grey-brown.
Maximum ionagar: mycelium rather more compact and dense.

Strands absent, and droplets becoming larger and coloured towards the centre of the colony. Undersurface soft brown with colourless margin, 4mm. wide.

APPENDIX B

The following reagents were used:-

1. 0·5g. gum guaiac in 30ml. methylated spirit.
2. 2% phenol plus a few drops of aniline.
3. 10% ammonium hydroxide.
4. Aniline.
5. 10% potassium hydroxide.
6. 40% formalin.

Positive results were obtained with two of the reagents only.

Fungus	Isolate	10% NH_4OH	10% KOH
L. elegans	1	PP	P
" "	52	PP	P
" "	63	PP	P
" "	67	PP	(P)
" "	77	PP	P
" "	81	(P)	(P)
B. luteus	19	-	-
" "	44	-	-
" "	74	(P)	-
" "	79	(P)	(P)
" "	83	(P)	-
B. bovinus	42	-	-
" "	70	-	-
" "	73	(P)	-

B. bovinus	78	-	-
B. variegatus	50	P	(P)
" "	55	P	P
" "	72	P	P
" "	82	P	(P)

(P) pale pink

P pink

PP deep pink

APPENDIX C

Analyses of variance.

Table	Fungus	Source	Mean Sq.	F	P
10	B. elegans	starch x gl.	8·42	280	0·001
		error	0·03		
10	B. elegans	pectin x gl.	8·27	82·7	0·001
		error	0·10		
10	B. elegans	pectate x gl.	0·33	2·5	n.s.
		error	0·12		
10	B. elegans	C.M.C. x gl.	6·51	130	0·001
		error	0·05		
10	B. variegatus	starch x gl.	24·61	9·5	0·025
		error	2·58		
10	B. variegatus	pectin x gl.	2·14	7·4	0·05
		error	0·29		
10	B. variegatus	pectate x gl.	0·07	0·3	n.s.
		error	0·23		
10	B. variegatus	C.M.C. x gl.	4·19	210	0·001
		error	0·02		
12	B. elegans	a.as x vits	1·50	4·2	n.s.
		error	0·36		
12	B. luteus	a.as x vits	12·25	68·1	0·001
		error	0·18		
12	B. bovinus	a.as x vits	6·00	20·0	0·005
		error	0·3		
12	B. variegatus	a.as x vits	0.05	0·2	n.s.
		error	0·23		
15	B. elegans	thiamin x vits	0·02	2·0	n.s.
		error	0·01		

15	B. luteus	thiamin x vits	0·54	0·2	n.s.
		error	0·22		
15	B. bovinus	thiamin x vits	0·28	0·2	n.s.
		error	0·15		
15	B. variegatus	thiamin x vits	0	0	n.s.
		error	0·09		
16	B. elegans	max. x roots	15·11	12·0	0·005
		error	1·26		
16	B. luteus	max. x roots	1219·77	44·4	0·001
		error	27·42		
16	B. bovinus	max. x roots	1362·26	354	0·001
		error	3·85		
16	B. variegatus	max. x roots	546·31	38·0	0·001
		error	14·37		
17	B. luteus (19)	max. x roots	381·23	65·0	0·001
		error	6·08		
17	B. luteus (44)	max. x roots	54·71	109	0·001
		error	0·05		
17	B. bovinus	max. x roots	1793·51	162	0·001
		error	11·08		