~~THE~~ PHYSIOLOGY OF [CERTAIN STERILE SEPTATE] FUNGI ASSOCIATED WITH ~~THE~~ ECTOMYCORRHIZAE OF **Pinus sylvestris**

by

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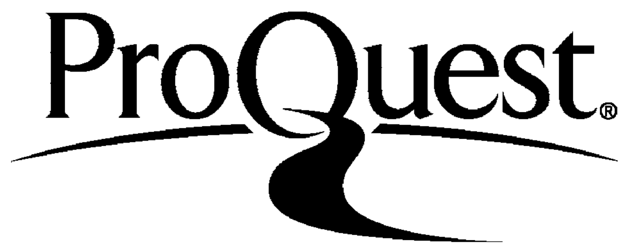

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ABSTRACT

Ninety-one root isolates were isolated from the ectomycorrhizal roots of *Pinus sylvestris*. *Boletus variegatus* 1 and *Boletus luteus* were isolated from sporophores.

Electron micrographs showed the dolipore septum to be present in *Boletus luteus*, but a simple pore occurred in the septum of root isolate M1.

Boletus luteus and some root isolates caused the dichotomy of the secondary laterals of *Pinus sylvestris* seedlings grown on Pachlewski agar slopes. 10^{-6}M indole-3-acetic acid had a similar effect.

Attempts to synthesize ectomycorrhizae *in vitro* between *Pinus sylvestris* and root isolates, *Boletus variegatus* 1 or *Boletus luteus* were of limited success. Several established techniques and modifications of them were used.

However many root isolates, also *Boletus variegatus* 1, were significantly stimulated by live excised tomato roots, this being a characteristic of ectomycorrhizal fungi, although *Boletus variegatus* 1 and the root isolate M13 reacted differently to exudates, extracts and homogenates of excised tomato and pine seedling roots.

Specific M13 growth stimulatory fractions of excised tomato and pine seedling root preparations were obtained by paper chromatography using two different solvent systems. There were no specific *Boletus variegatus* 1 growth stimulatory fractions.

A number of organic compounds were positively identified by paper chromatography to be present in the excised tomato root preparations. Riboflavin had similar fractions as the M13 growth stimulator(s) in both solvent systems. The identity of these stimulatory spots as riboflavin was confirmed by the use of the

Mass Spectrometer. Also its effect on the growth of M13 was determined.

The nutritional requirements of M13 were similar to those of several unnamed ectomycorrhizal fungi, but differed from the requirements of certain named ectomycorrhizal fungi, for example *Boletus variegatus*.

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REVIEW OF THE LITERATURE.

1. THE SYMBIOTIC ASSOCIATION.

a) Morphological.

Ectomycorrhizae occur very widely in nature, and trees bearing ectomycorrhizae are found more frequently than trees which do not. They are produced as a result of a symbiotic relationship between a fungus, which is usually a Basidiomycete and forest trees, which in this country belong to the families Pinaceae, Betulaceae and Fagaceae.

An ectomycorrhiza consists basically of a stele of host tissue surrounded by the endodermis. Outside the endodermis is the cortical region between the cells of which the hyphae of the symbiotic fungus pass to form the Hartig net. Outside this region is the mantle which consists entirely of fungal tissue of closely interwoven and compact fungal hyphae, and it is this mantle and the enlarged cortical cells which give the root the appearance of being swollen. The infection zone occurs behind the growing tip and in front of the region where the primary cortex deteriorates with the maturation of the root, and has been termed the Mycorrhizal Infection Zone or MIZ (Marks and Foster, 1973).

Ectendomycorrhizae occur when the fungal hyphae instead of passing only intercellularly through the cortex, also penetrate the cells intracellulary.

Hofsten (1969), after studying an ectendomycorrhiza found that the typical dolipore septum, present in all Basidiomycetes, occurred in the hyphae of the Hartig net.

The actual distinction between these two types of mycorrhizae is still not clearly understood. It has been suggested that they may be produced by two different fungi, conditions in the soil favouring one type of fungus rather than the other (Mikola, 1965). Alternatively ectendomycorrhizae may result from secondary invasion by a weakly parasitic fungus (Hacskaylo, 1957) or one which normally produces ectomycorrhizae (Wilcox, 1971).

Morphologically pine ectomycorrhizae are distinct and differ, for example, from those found on beech. The ectomycorrhizal roots of pine are much stunted, usually swollen and often dichotomously branched, either simply or more profusely to form a coralloid structure or even a tubercle. In beech the young non-suberized roots become infected and form racemosely branched ectomycorrhizal systems.

There has been published a list of higher fungi which form regular associations with coniferous and other tree species (Trappe, 1962, 1963), although these associations have not always been confirmed by _in vitro_ syntheses. These latter procedures are basically methods to determine whether ectomycorrhizal formation will occur under sterile conditions in a controlled environment (Melin, 1921; Norkrans; 1949; Hacskaylo,

1953; Fortin, 1966; Pachlewski, 1967; Pachlewska, 1967; Pachlewski and Pachlewska, 1967).

These ectomycorrhizal fungi usually belong to the Basidiomycetes and are septate, often with clamp connections. Some Ascomycetes have been isolated, and one, Cenococcum graniforme, is known to occur widely in the ectomycorrhizal roots of forest trees. Also a number of truffles have been shown to form ectomycorrhizae in vitro, e.g. Tuber brumale with Pinus nigra (Fontana and Fasolo Bonfante, 1971). Very little work has been done with these ascomycetous fungi, because of lack of success in growing possible species in pure culture, this being strong evidence that they are not saprophytic fungi (Trappe, 1971). Trappe says that since Cenococcum graniforme appears to be anatomically similar to Elaphomyces species, it could perhaps be the vegetative stage of Elaphomyces anthracinus.

It is possible to isolate the symbiotic fungus from the ectomycorrhizal root (Zak and Bryan, 1963; Zak and Marx, 1964; Lamb and Richards,1970; Marx et al., 1970[b]; Zak, 1971[a]; Peña-Cabriales and Valdés-Hgo, 1974). After sterilizing the outside of an ectomycorrhizal root and transferring it to a suitable nutrient medium the fungus(i) will grow out of the root after a period of 2 to 6 weeks, when it can be kept in pure culture, where it usually remains sterile . It has been possible to match some of these fungi with known ectomycorrhizal fungi on the

basis of rate of growth and character of mycelial mat, but a large number of such root isolates still remain unidentified (Zak and Bryan, 1963).

b) Biochemical aspects.

Certain biochemical changes occur in the host root after the formation of ectomycorrhizae.

The amino acid content of the root alters (Krupa *et al.*, 1973; Krupa and Bränström, 1974). Arginine and citrulline accumulate in the free pool of the cells of non-mycorrhizal roots of *Pinus nigra* and *Corylus avellana*, but in the ectomycorrhizal root system there was a general decrease in the amino acids, followed by an increase in glutamine and asparagine. Also there was a decrease in the free arginine present in *Boletus variegatus* hyphae when it formed the symbiotic relationship with *Pinus sylvestris* and so arginine synthesis in *Boletus variegatus* is probably repressed by the host. It is suggested that the fungus uses the host arginine pool to form a more efficient protein synthesis system in both the host and the fungus.

Antibiotics are produced by ectomycorrhizal fungi *in vitro* (Sasek and Musilek, 1967). They can also be extracted from ectomycorrhizal roots formed by *Cenococcum graniforme* on *Pinus strobus*, *P. resinosa*, and *Picea abies* (Krywolap *et al.*, 1964) and from ectomycorrhizae formed by *Leucopaxillus cerealis* on *Pinus echinata*. These antibiotics are important

in the resistance of ectomycorrhizal roots to *Phytophthora cinnamomi*, a pathogen of feeder roots (Marx and Davey, 1969a and 1969b).

After studying the rhizosphere of morphologically different ectomycorrhizal roots it seems that there is a variation between them, in both number and types of bacteria and fungi present (Neal *et al.*, 1964; Foster and Marks, 1967). The differences between these morphologically different ectomycorrhizas are thought to be due, directly or indirectly, to different fungal symbionts.

Zak (1967) observed a nematode belonging to the genus *Meloidodera* associated with two, morphologically distinct types of ectomycorrhizas, along a 50.8cm *Pseudotsuga menziesii* root. More Actinomycetes and bacteria, including bacteria able to reduce methylene blue, ferment glucose and promote ammonification, are found in ectomycorrhizal than non-ectomycorrhizal root rhizospheres of *Betula alleghaniensis* (Katznelson *et al.*, 1962). These bacteria probably all belong to one group and are also present in the mantle of the ectomycorrhizal roots of *Pinus radiata*, the symbiotic fungus being *Boletus granulatus*. One such bacterium, R1-2, is stimulated by the exudates of *B. granulatus*, but not by *Pinus radiata* roots. R1-2 also develops well in a medium suitable for nitrogen-fixing bacteria (Rambelli *et al.*, 1972).

Therefore these biochemical changes which occur after ectomycorrhizal formation, possibly produce

an alteration in the rhizosphere of the host root, such that a new microflora forms and hence a mycorrhizosphere.

c) Physiological.

It is known that pine seedlings inoculated with an ectomycorrhizal fungus show improved growth (Finn, 1942; Theodorou and Bowen, 1970), even if ectomycorrhizal formation, as such, does not occur. Also most benefit is obtained by the pine seedling if it is inoculated with a potential ectomycorrhizal fungus which has been isolated from the same tree species (Lamb and Richards, 1971).

More nutrients are known to be taken up by ectomycorrhizal than non-ectomycorrhizal plants growing under nutrient-poor conditions. This has been shown to occur in pine seedlings, where there was an increase of 86% nitrates, 234% phosphates and 75% potassium in ectomycorrhizal plants, compared with non-ectomycorrhizal plants (Hatch, 1937). This has been confirmed for phosphate (Kramer and Wilbur, 1949) and for ammonium, calcium, glutamic acid, phosphate and sodium (Finn, 1942; Melin and Nilsson, 1950, 1952, 1955; Melin et al., 1958). Other results based on experiments with excised beech ectomycorrhizae have shown that most of the phosphate is stored in the mantle, unless the phosphate in the substrate is reduced and then it passes into the host tissue (Harley and McCready

1950, 1952).

The ectomycorrhizal root, as well as the mycelial strands extending from these roots, increase the absorbing surface, hence there is likely to be an increase in the amount of water and minerals which can be taken up by these roots. It is known that ions exist in the soil, either adsorbed onto the surface of colloids, or as part of the soil mineral solutions. These colloidal surfaces are lacking in infertile soil and so most of the ions exist as part of the soil mineral solutions and tend to be lost to the plant by leaching. Extensive mycelial strands must be of advantage in nutrient uptake in such situations. Mycelial strands attached to ectomycorrhizal roots (as well as fungi and bacteria already in the soil) are also known to produce acids which dissolve the silicates in the soil, the released ions being absorbed by the mycelial strands and the ectomycorrhizal roots (Voigt, 1971).

During a period of summer drought, there was an increase in the number of pine seedlings which survived after having been inoculated with the ectomycorrhizal fungi, Rhizopogon luteolus and Boletus granulatus (Theodorou and Bowen, 1970).

It has been further suggested that the ectomycorrhiza also protects the root from attack by parasitic fungi, possibly by serving as a physical barrier to infection, by secreting antibiotics

into the soil, by using the surplus carbohydrates and so reducing the attractiveness to root pathogens, or by favouring the rhizosphere organisms which protect the root from parasitic fungi. There is now some experimental evidence to support this hypothesis (Zak, 1964, 1971[b]; Marx, 1971, 1973).

Monoterpenes and sesquiterpenes are both relatively abundant constituents of ectomycorrhizal root systems of Pinus sylvestris and as they are known to inhibit the growth of Phytophthora cinnamomi and Fomes annosus, it has been suggested that they play an important part in disease resistence (Krupa and Fries, 1971; Krupa and Nylund, 1972).

2. FORMATION OF ECTOMYCORRHIZAE.

Ectomycorrhizae are formed in the field on contact between an actively growing root and a compatable fungus. The fungus may origionate initially from spores in the soil, or from hyphae from a residual mycelium, or by the progression of hyphae from the Hartig net of established ectomycorrhizae (Robertson, 1954).

A number of factors directly affect the formation of the symbiotic relationship between plant and host, and of these the most discussed have been soil nutrient status and acidity and light.

Ectomycorrhizae tend to develop more frequently in acid soils (Marx and Zak, 1965), and cultured ectomycorrhizal fungi have their optimum growth in an acid medium.

It has been suggested that since the surface of the root is continually being recharged with hydrogen ions, this may act as a buffer against any change in soil pH, so maintaining the root surface in an acid environment (Voigt, 1962). Other experiments also indicate that ectomycorrhizal development is not dependant directly on pH, but on some other external factor, for example nitrification. When the nitrate content of the soil is increased, no matter what the pH is, then the number of ectomycorrhizae decreases (Richards, 1961; Richards and Wilson, 1963). High phosphate as well as high nitrate levels also reduce ectomycorrhizal formation (Fowells and Krauss, 1959). High levels of nitrates and phosphates in fully illuminated plants, also a low light intensity suppressed ectomycorrhizal formation on pine roots (Bjorkman, 1942), as also did strangulation of the stems of young pine trees (Bjorkman, 1944).

Further results obtained by Bjorkman (1970) suggested that

the levels of reducing substances in the pine needles were constant at the different soil nutrient concentrations, but were lowered in the needles and roots of shaded plants and in the roots of plants grown in full and half daylight, fertilized with nitrates. Higher levels were obtained in roots of plants grown with added phosphates and also with an increase in the length of the photoperiod. Bjorkman concluded from these results that the suppression of ectomycorrhizal formation with a short photoperiod results from a low sugar content in the root. The effect of length of photoperiod was confirmed by Boullard (1961) and Hacskaylo (1969, unpublished but cited by Hacskaylo, 1973). Conflicting results were obtained by Mikola (1948) who found an increase in ectomycorrhizal formation with a decrease in length of the photoperiod, using plants of Betula pubescens which had been inoculated with Cenococcum graniforme.

Experiments by Handley and Sanders (1962) using exactly the same experimental conditions as Bjorkman (1942), but with non-ectomycorrhizal pine seedlings, have indicated that excess soluble reducing substances in the ectomycorrhizal roots may occur as a result of ectomycorrhizal formation, possibly due to the accumulation of these soluble reducing substances in the fungal mycelia. Also Lister et al. (1968) found the same levels of sucrose, glucose and fructose present in both ectomycorrhizal and non-ectomycorrhizal roots.

Meyer (1962) also obtained similar results to Bjorkman (1942), but in some of his experiments there was an increase in ecto-mycorrhizal frequency in a nutrient rich soil, instead of a nutrient deficient soil. Also on the addition of nitrates and phosphates to the soil, there was no reduction in the numbers of

ectomycorrhizae, and in fact some cases show an increase. Therefore it may not be the actual concentration of the phosphates and nitrates in the soil which affect ectomycorrhizal formation, but the balance between them (Slankis, 1971).

Harley (1969) does not accept Bjorkman's origional hypothesis and he points out that carbohydrates would be a very non-specific stimulus to ectomycorrhizal infection anyway. It may be that, although specific light and nutrient supplementations are necessary, the actual response depends on, for example, a specific soil nutrient status or a specific host/fungus combination. This would account for the different results obtained by various workers using different soil nutrient states and different host/fungus combinations. For example Mikola (1948) who obtained an increase in ectomycorrhizal formation at low light intensities used Betula pubescens combined with Cenococcum graniforme, whereas Bjorkman (1970) who obtained an increase in ectomycorrhizal formation at higher light intensities used Pinus sylvestris seedlings associated with either Boletus bovinus or Boletus subtomentosus. Also the soils used by Mikola and Bjorkman were obtained from different sources and hence were not nutritionally identical.

The work of Pelham and Mason (1975) indicates the importance of each host/fungus combination in requiring a specific nutrient status. Seed of birch (Betula verrucosa) obtained from Scotland showed no difference in the quantity of ectomycorrhizae obtained at high and low phosphate concentrations, whereas seed from Latvia, sown in the same soil produced more ectomycorrhizae at low phosphate concentrations.

Further results were obtained by Pelham and Mason (1975),

using various sources of both birch seed and the symbiotic fungus *Amanita muscaria*. One isolate of *Amanita muscaria*, from pine trees in America, would not form ectomycorrhizae with birch, whereas isolates collected from birches in Britain did. There was also variation in the number of short roots which became ectomycorrhizal, 60-70% with birch seed provenance 1 and 2, but only 10% with seed provenance 3. Pelham and Mason suggested that there is probably a genetical control of ectomycorrhizal formation, similar to that found with the legume/*Rhizobium* complex. They also suggest that it might be useful in forestry practice to select trees and fungal partners. Similar results to those of Pelham and Mason, were obtained by Wright and Ching (1962) using *Pseudostuga menziesii* seedlings, from many different seed sources, grown in forest nurseries in unsterilized soils.

Ectomycorrhizal formation may be influenced by toxins produced by pine roots (Foster and Marks, 1966, 1967; Hillis *et al.*, 1969) or by associated macroflora, for example grasses (Theodorou and Bowen, 1971) and *Calluna* sp. (Handley, 1963, cited by Harley, 1969).

The highest seasonal frequency of ectomycorrhizal development of pine trees growing in forest stands is in the spring, with a decrease in the summer, probably due to the reduced rainfall and higher transpiration rates. An increase occurs again in the autumn and another decrease in the winter, when presumably low temperatures suppress root and fungal growth (Hacskaylo, 1957). Hence both temperature and availability of water appear to affect the formation of ectomycorrhizae.

Rhizosphere activity in general may be important in ectomycorrhizal formation. The growth of ectomycorrhizal fungi in

the rhizosphere of other plants may be important to the survival of these fungi in the field. It is known that *Rhizopogon luteolus* will colonize the sterile roots of the non-host plant, *Lolium perenne* (Theodorou and Bowen, 1971). Rhizosphere micro-organisms may play some role in the formation of ectomycorrhizae *in vivo*, and it has been reported that endomycorrhizal infection by *Endogone* sp. could only be achieved aseptically in the presence of *Pseudomonas* sp. (Mosse, 1962).

Conditions necessary for ectomycorrhizal formation are specific for each host/fungus combination, as indicated by Pelham and Mason's work (1975). Hence the inability to synthesize ectomycorrhizae with certain host/fungus combinations *in vitro* may not be due to an innate inability of the fungus to form ectomycorrhizae, but because the conditions during the experimental period are too far removed from those required by that specific host/fungus combination *in vivo*. The inability, therefore, of a specific fungus to form ectomycorrhizae during *in vitro* synthesis experiments is not always definite proof of the inability of that fungus to form ectomycorrhizae under natural conditions. Therefore negative results from *in vitro* synthesis tests, which are used by many workers to prove whether a fungus is ectomycorrhizal or non-ectomycorrhizal must be viewed with caution.

a) *Hormones.*

Ectomycorrhizal fungi are known to liberate substances extracellularly. In the ectomycorrhizal root, the fungus not only passes nutrients from the soil to the host plant, but also produces and probably translocates

growth hormones, like auxins, cytokinins, gibberellins and growth regulating B-vitamins. This results in a higher concentration of hormones within the plant than usual and thus influences the growth and development of the host (Slankis, 1973).

Cell free filtrates from Boletus variegatus and Boletus luteus cause the dichotomy of pine root similar to that obtained during ectomycorrhizal formation, as also does indole-3-acetic acid (IAA), other indoles and naphthalene acetic acid (NAA) at suitable concentrations. NAA is more effective than IAA. As the concentration of IAA is increased root hairs disappear, the cortical cells gradually show radial expansion and the degree of dichotomy also increases (5-10mg IAA/litre), until at a higher concentration, structures similar to tubercle ectomycorrhizas form (10-20mg IAA/litre) (Slankis, 1948, 1949, 1950, 1951, 1958,1963).

In order to maintain this dichotomous development, either fungal exudate or synthetic auxin has to be supplied every other week, otherwise the swollen tips begin to elongate and the ectomycorrhizal characteristics disappear. Therefore there must be a continuous production of IAA or similarly acting growth hormone in the roots, perhaps produced by the fungus (Slankis, 1973).

It is generally assumed that ectomycorrhizal fungi are responsible for the morphological changes which occur in the root (Gogala, 1971). It is known that fungal auxins are transported to the mother root from ectomycorrhizas, and eventually to the rest of the root

system, and that exogenous auxin absorbed by one region of the root system will affect the rest of the root system (Slankis, 1973).

The amount and time of production of these hormones varies between fungal species (Moser, 1959; Ulrich, 1960), for example *Boletus variegatus* will produce IAA in detectable amounts after 3 days, whereas *Amanita frostiana* requires 7 months. This might account for variations in numbers of dichotomies in ectomycorrhizal pine root systems.

Cytokinin is produced by the ectomycorrhizal fungus *Rhizopogon roseolus* (Miller, 1971; Crafts and Miller, 1974) and zeatin has been isolated in crystalline form, but it has not been determined whether cytokinins are actually produced in the ectomycorrhizal root. Also gibberellic acid and a complex of compounds thought to be gibberellic acid esters were produced in a culture medium of *Boletus edulis* (Gogala, 1971). There is no evidence that these growth hormones are involved in ectomycorrhizal synthesis or that they affect root morphology.

b) *Root exudates.*

Roots are known to exude substances into the medium in which they are growing, and it is known that these substances do in fact affect the growth of soil microorganisms, for the soil immediately adjacent to the root surface, i.e. the rhizosphere, contains more and often different microorganisms compared with soil

distant from the root.

The main substances exuded from coniferous roots include carbohydrates, amino acids and organic acids (see Table 1 below).

Table 1.

Amino acids, organic acids and carbohydrates exuded from coniferous roots. (Slankis *et al.*, 1964; Smith, 1969, 1970; Bowen, 1969)

Amino acids	Organic acids	Carbohydrates
Aspartic acid	Acetic	Glucose
Glycine	Oxalic	Sucrose
y-aminobutyric acid	Succinic	Rhamnose
Valine	Malonic	Ribose
Proline	Citric	Arabinose
Alanine	Oxaloacetic	
Lysine	Glycolic	
Arginine	Malic	
Phenylalanine	Shikimic	
Serine	cis-aconitic	
Threonine		
Asparagine		
Methionine		
Tyrosine		
Glutamine		
Glutamic acid		
Leucine/isoleucine		

Certain unidentified substances are known to be exuded by these roots, also the vitamin, niacin has been identified in the root exudate of *Pinus radiata* (Smith, 1969).

Variation in the compounds released and also the quantity does occur between different coniferous species (Smith, 1969). There may be some variation due to the age of the tree (Smith, 1970) and also to soil nutrient status (Bowen, 1969). It has been shown that the loss of amides and amino acids from the roots of *Pinus radiata* is 2½ times greater in phosphate deficient soils than in normal soils, and 10 times greater in nitrogen deficient soils (Bowen, 1969).

Of the unidentified compounds exuded from pine roots, one is termed the M-factor, which is responsible for stimulating the growth of a number of ectomycorrhizal fungi (Melin, 1953, 1954, 1962, 1963; Melin and Das, 1954). This substance or substances is/are also known to be present in exudates of tomato, *Lepidium sativum* L., *Medicago sativa* L., *Cannabis sativa* L., *Pisum sativum* L., and *Triticum aestivum* L. A few ectomycorrhizal fungi are completely heterotrophic for this factor, e.g. *Russula xerampelina*, *Pholiota cuperata* and *Lactarius helvus*, and will only grow in the presence of roots which produce it. On the other hand *Boletus variegatus*, *Rhizopogon roseolus*, *Lactarius rufus*, and *Lactarius mitissimus* are only partially deficient for it, the M-factor increasing their growth in the first stages of development, but they eventually give the same

maximum mycelial yields as the control. This stimulation appears to be unique to ectomycorrhizal fungi, for although Boletus variegatus, Boletus subtomentosus and Boletus scaber are all stimulated by the presence of pine roots, Psalliota arvensis, a litter decomposing fungus is not (Lundeberg, 1960). Also the non-ectomycorrhizal Hymenomycetes Hypholoma fasiculare, Clitocybe infundibuliformis, Collybia butyracea, Trametus versicolor, and Paxillus atromentosus are not affected by tomato roots, and Marasmius alliaceus is inhibited by them (Ferry, 1967).

The M-factor appears to be exuded from the root and not contained within it, since the same response is obtained with excised pine roots enclosed within celluloid bags, and in fact stimulation of these fungi did occur when pine root exudate was collected and applied to the fungus (Melin, 1962). The maximum effect of the M-factor on Boletus variegatus occurs at a concentration of 5-10 units* of cultured pine root exudate. Concentrations of more than 20 units exert an inhibitory effect and at 40 units the amount of inhibition was 70% over a period of 8 days (Melin, 1963).

Autoclaving the exudate of both pine and tomato roots gives a greater increase in growth stimulation, and Melin (1963) suggested that this was because a heat labile factor which passes into the root exudate and normally

* One unit of exudate is equal to the amount collected from 1mg dry weight of root for 6 days at $4^{o}C$.

causes the gradual inactivation of the M-factor, was destroyed. From the results of other experiments Melin (1962) suggests that the M-factor consists of two parts, one being soluble and the other insoluble in water, the insoluble component only being available to the fungal hyphae by the enzymic activity of the fungus.

Melin proposes that the M-factor is closely bound up with the root cell metabolism, but it appears that it is not a B-vitamin, purine or pyrimidine base or an amino acid present in casein hydrolysate. These substances will not replace the root effect. The root effect is however negated in the presence of adenine or its derivatives (Melin, 1959). In a report to the Swedish Council of Natural Science (unpublished), Nilsson (1960) has stated that the diffusible M-factor could be replaced by diphosphopyridine nucleotide.

An unknown root exudate factor is known to stimulate spore germination of ectomycorrhizal fungi, either by accelerating their germination in the presence of roots e.g. spores of Boletus luteus or by inducing spore germination e.g. spores of some species of Russula (Melin, 1962). This spore inducing factor is also heat stable (Ferry, 1967).

Both pine and tomato roots exude growth inhibitory substance(s) (Melin, 1962, 1963). However these substance(s) do not seem to pass easily from live roots, more being extracted from freeze dried or crushed roots. If freeze dried or crushed pine roots are autoclaved before putting them into water, a growth promotion is obtained

which is similar to untreated roots, suggesting that the inhibitory factor(s) may be heat labile (Melin, 1963).

These results suggest that intact roots also produce an inhibitor(s) which at certain concentrations may counteract the M-factor (Melin, 1963). Possibly tomato root inhibitor(s) prevent the ectomycorrhizal fungus from entering the roots altogether, whereas pine root inhibitor(s) confine the fungus to definite parts of the infected roots. More inhibitor(s) is produced in the main axis of excised pine roots (cultured for 6 months), since fungal development only occurred around the youngest laterals of these roots (Melin, 1962). Inhibitor(s) may also limit certain ectomycorrhizal fungi to certain tree species. There is evidence that the inhibitor(s) is volatile or otherwise labile as its effect is lost after a few days.

Monoterpenes and sesquiterpenes occur naturally in both ectomycorrhizal and non-ectomycorrhizal roots of Pinus sylvestris seedlings (Krupa and Fries, 1971). The effect of some of these compounds on the growth of ectomycorrhizal fungi was determined by Melin and Krupa (1971). Inhibition occurred with both a mixture of and individual monoterpenes and sesquiterpenes. Hence it could be that these compounds are the inhibitor(s) found by Melin, since their concentration within the root is just enough to restrict the growth of the ectomycorrhizal fungus within the host root, to establish an ectomycorrhizal relationship. These volatile substances are not fungicidal, and once removed, the fungus is able

to continue growing again.

All of these root exudates, identified and unidentified, are produced from areas around the root tip, i.e. from regions of high meristematic activity and cell elongation (Slankis et al., 1964), and so it is in this region that microorganisms will react to the changed environment. For example malonic acid (see Table 1) which is a competitive inhibitor of succinic dehydrogenase may influence the oxidative reactions of microorganisms in the rhizosphere. Shikimic acid (see Table 1) is an important intermediate in microorganisms in the pathway concerned with the synthesis of phenylamine and tyrosine (Quastel and Wooldridge, 1928; Davis, 1951, both cited by Slankis et al., 1964) and malic acid (see Table 1) may relate to the observed depression of the microflora of Brassica juncea in the soil (Andal et al., 1956). The rhizosphere microorganisms will in turn influence, by their metabolic activities, other microorganisms and these in turn the roots.

Recent investigations have shown that different plants tend to select a specific fungal microflora in their rhizospheres (Rambelli, 1973), and specific ectomycorrhizal associates are extreme examples of these. After the formation of ectomycorrhizae the tree root rhizosphere will undergo a change, since the symbiotic fungus in the mantle will metabolise some of the root exudates, these then passing out into the soil as fungal metabolites (Rambelli, 1973).

3. GROWTH REQUIREMENTS OF ECTOMYCORRHIZAL FUNGI.

The fungus in the root probably depends entirely on the host plant for its source of carbohydrates and many experiments have shown that the carbohydrates from the host are transferred to the fungus (Melin and Nilsson, 1957).

Glucose is known to be the best carbon source for ectomycorrhizae, grown in pure culture (Melin, 1925; How, 1940; Norkrans, 1950; Ferry, 1967). They can also utilize other carbon sources, for example Cenococcum graniforme grows equally well on sucrose and maltose and can utilize dextrin, starch and mannitol (Mikola, 1948) and also mannose, trehalose, cellobiose and α dextrin (Keller, 1952). Other ectomycorrhizal fungi can use inulin and starch (Palmer and Hacskaylo, 1970; Lamb, 1974) as well as the previous mentioned carbohydrates. Adaptive growth on carbon sources can occur when glucose is added to the medium at a concentration of 0.1g carbon per litre (Lamb, 1974).

Cellulose can be used as a source of carbon by a few of these fungi. Tricholoma fumosum which forms ecto- and ectendomycorrhizae with pine will significantly utilize cellulose (Norkrans, 1950) as also will Boletus subtomentosus, Boletus luteus, Boletus variegatus and Amanita citrina, although to a far lesser extent than wood or litter-destroying fungi (Lyr, 1963). When glucose is added as a starter (0.5g per litre), Tricholoma imbricatum and Tricholoma vaccinum can give slight growth in the presence of cellulose (Norkrans, 1950), this also being noted to occur with some of the 21 isolates used by Lamb (1974) It is proposed (Norkrans,1950) that

inducible enzyme synthesis is involved here.

Hacskaylo (1973) criticizes these results. He says that they may be misleading or incomplete, since impurities in the test solutions may have affected the ability of these fungi to utilize the carbon sources. Also the detection of cellulolytic enzymes in ectomycorrhizal fungi depends on the sensitivity of the assay and repeated results vary considerably.

Evidence for lignin utilization is meagre and for the most part indirect. Boletus subtomentosus (a mycorrhizal-former with Pinus montana) and Lactarius deliciosus (a mycorrhizal-former with Pinus sylvestris and Picea abies) both produce large quantities of polyphenoloxidase enzymes, when grown on gallic and tannic acid media (Lindeberg, 1948). Also Tricholoma fumosum is able to utilize lignin in Glyceria straw (Norkrans, 1950).

Boletus variegatus can grow on ground-up forest litter which has been previously leached with water (Melin, 1946) and also forest humus if glucose is present (Melin, unpublished).

Nitrogen nutrition in ectomycorrhizal fungi is very similar to other non-ectomycorrhizal Basidiomycetes, growth being better on ammonium tartrate, propionic acid amide, glycocoll, asparagine, casein hydrolysate and peptone rather than nitrates (Rawald, 1963).

Growth of Lactarius deliciosus increases when a mixture of 18 amino acids are present in the medium together with an ammonium source. These amino acids are very similar to those found in casein hydrolysate (Melin and Norkrans, 1948). Certain species of Tricholoma are inhibited by some amino acids when added individually to the nutrient medium (Norkrans, 1950).

Casein hydrolysate generally gives the greatest increase in growth of all the amino acid mixtures.

Growth promotion is also obtained with extracts of certain Hymenomycete sporophores (Melin and Norkrans, 1948), but this is thought to be due in part, to amino acids.

Thiamin heterotrophy occurs widely in many soil-inhabiting and wood-destroying Basidiomycetes (Robbins and Kavanagh, 1942) as well as in ectomycorrhizal Basidiomycetes (Chudjakov and Woznjakowskaja, 1951; Melin, 1953; Rawald,1962). From 15 species of ectomycorrhizal fungi which were tested by Melin, most of them were either partially or totally dependant on thiamin for their growth. Different isolates of the same species vary in having different requirements, and different environmental conditions are known to alter these requirements. Melin found that most of the fungi he used required both thiamin moieties i.e. thiazole and pyrimidine, but a few required only the pyrimidine moiety.

Tricholoma imbricatum is partially heterotrophic for pantothenic acid (Norkrans, 1950), *Lactarius deliciosus* is partially heterotrophic for nicotinamide (Melin, 1953) and *Rhizopogon roseolus* is partially heterotrophic for inositol (Melin and Lindeberg, 1939).

The effect of short chain fatty acids on the growth of some ectomycorrhizal fungi (species of *Boletus*) and saprophytic Hymenomycetes (species of *Marasmius*) is to strongly inhibit their growth, *Boletus* species being much more sensitive than *Marasmius* species (Lindeberg and Lindeberg, 1974).

The optimum growth of ectomycorrhizal fungi occurs in an acidic environment. *Boletus elegans*, *Boletus luteus*, *Boletus*

bovinus and *Boletus variegatus* reach their optimum growth at pH 5·0 (Ferry, 1967).

The temperature at which optimum growth occurred in the above four species of *Boletus* was at 25°C (Ferry, 1967), although other ectomycorrhizal fungi may have their optima either above, e.g. *Amanita rubescens* and *Suillus punctipes* at 29°C, or below, e.g. *Rhizopogon roseolus* at 13°C (Hacskaylo *et al.*, 1965).

INTRODUCTION

The main purpose of this investigation was to obtain more information concerning the physiology of the fungi which are associated with the ectomycorrhizas of *Pinus sylvestris*.

Initially an attempt was made to synthesize ectomycorrhizas on the roots of *Pinus sylvestris* *in vitro*, with certain root isolates and with *Boletus luteus* and *Boletus variegatus* 1, two named ectomycorrhizal fungi. Various established techniques were used, in both sterile and non-sterile environments, in order to ascertain whether these fungi were involved in ectomycorrhizal formation. However, these synthesis experiments were not successful with any of the root isolates tested and,since one of these root isolates, M13, was stimulated by a certain unknown growth factor(s) present in both excised tomato root and pine seedling root preparations, Melin's M-factor experiments were repeated and extended, in order to obtain more information concerning the nature of this growth factor(s) and also its effect on the growth of both M13 and *Boletus variegatus* 1 *in vitro*.

The nutrition of many named ectomycorrhizal fungi *in vitro* is known to differ somewhat from non-ectomycorrhizal fungi. Since data concerning unnamed ectomycorrhizal fungi is rather scarce, experiments were undertaken in order to determine whether the nutrition of one of these root isolates, M13, was similar to the results already obtained for named ectomycorrhizal fungi. Also to determine whether M13 and three other root isolates were able to utilize the lignin and cellulose present in pinewood sawdust.

It was hoped that these results, of both the M-factor and the nutritional experiments, might give some information concerning the ecological status of M13.

Most of the ectomycorrhizal fungi reported in the literature

belong to the Basidiomycetes, although a few are Ascomycetes. In order to ascertain to which group these isolates belong a few of the root isolates were selected and their ultra-structure examined for the presence or absence of a dolipore septum, this being characteristic of fungi belonging to the Basidiomycetes e.g. Boletus luteus.

MATERIALS AND METHODS

ISOLATION OF THE FUNGI FROM ECTOMYCORRHIZAL ROOTS OF PINUS SYLVESTRIS AND SPOROPHORES.

Ectomycorrhizal roots were obtained from Brandon Forest, near Thetford, Norfolk and from Angley Woods, near Cranbrook, Kent. Two methods were used to isolate the fungi from the ectomycorrhizas.

Ectomycorrhizal roots were cut into 12 to 15cm lengths and washed in a sieve under the tap to remove all adhering soil, were rinsed in distilled water, and finally the ectomycorrhizal short roots were severed from the main root. These were then surface sterilized for 30 minutes in 100vols hydrogen peroxide, containing a drop of "Tween 80", and were then transferred, without washing, onto agar plates (16 roots per plate) containing the following media:-

1. Malt Extract Agar (MEA).

Malt extract (Oxoid)	15g
*Oxoid agar No.3	12g
Distilled water	to 1000ml

pH 5•5

*Later media contained Agar Agar (Solmedia).

2. Basic Ion Agar (BIA) and Basic Liquid Medium (BLM). (Melin et al., 1954)

Glucose	20g
Ammonium tartrate	0•5g
$MgSO_4 \cdot 7H_2O$	0•5g
KH_2PO_4	1•0g
$FeSO_4 \cdot 7H_2O$	5•0mg

2. (cont.)

$ZnSO_4 \cdot 7H_2O$	5•0mg
Thiamin HCl	0•05mg
Purified agar (Oxoid)	15g (only present in BIA)
Distilled water	to 1000ml

pH 5•0

3. <u>Taylor's Medium (selective for Basidiomycetes).</u>

<u>(Taylor, 1971)</u>

Glucose	10g
KH_2PO_4	1•0g
$(NH_4)_2SO_4$	1•0g
$MgSO_4 \cdot 7H_2O$	0•5g
Peptone (Oxoid)	1•5g
Thiamin HCl	100ug
Purified agar (Oxoid)	15g
Distilled water	to 1000ml

pH 5•5

Selective agents:-	Benomyl	5ug/litre
	Neomycin sulphate	50ug/litre
	Streptomycin sulphate	50ug/litre

These selective agents were added from stock solutions of 1 or 10mg/litre, suspended in 0•1% water agar and kept in the refrigerator.

4. <u>Modified Melin-Norkran's Medium.</u>

<u>(Marx, 1969)</u>

$CaCl_2$	0•05g
NaCl	0•025g
KH_2PO_4	0•5g
$(NH_4)_2HPO_4$	0•25g

4. (cont.)

$MgSO_4 \cdot 7H_2O$	0•15g
$FeCl_3$ (1%)	1•2ml
Malt extract (Oxoid)	3•0g
Thiamin HCl	100ug
Sucrose	10g
Purified agar (Oxoid)	15g
Distilled water	to 1000ml

pH 5•5

Analar reagents were used where available. All media were autoclaved at 15 p.s.i. for 15 minutes and 30ml was transferred aseptically to each sterile petri dish (9cm).

The inoculated plates were then incubated at 18°C in the dark.

The second method was similar to that used by Zak and Bryan (1963), Zak and Marx (1964) and Lamb and Richards (1970). The ectomycorrhizal roots were washed as before, and then root pieces 1•5 to 3•0cm long, bearing suitable ectomycorrhizae, were severed and placed in a screw cap polythene bottle containing 0•1% solution of "Teepol". They were hand-shaken vigorously for 3 to 4 minutes. The ectomycorrhizae were then transferred to a square of dental gauze and washed under a stream of running water to remove all traces of detergent, and finally the gauze was drawn together, so enclosing the roots, and tied in place using cotton thread. The bag was immersed in a 1% solution of sodium hypochlorite and continuously agitated for 10 minutes, was removed aseptically from the solution, and 2 litres of sterile water passed through it. The ectomycorrhizae

were then floated out aseptically onto sterile distilled water and severed from the main root with a sterile sharp scalpel. Finally they were transferred to agar plates containing the following media (30ml per plate):-

i) MEA (see page

ii) Hagem Medium.

(Modess,1941)

Glucose	5•0g
NH_4Cl	0•25g
$MgSO_4$•$7H_2O$	0•25g
KH_2PO_4	0•25g
Malt extract (Oxoid)	5•0g
$FeSO_4$•$7H_2O$	5•0mg
Purified agar (Oxoid)	15g
Distilled water	to 1000ml
Streptomycin sulphate	80ppm

pH 5•1

iii) Modified Hagem Medium.

(Modess, 1941)

As above (ii), with the addition of 1g/litre yeast extract (Oxoid).

pH 5•8

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

Twelve roots were placed in each sterile petri-dish and were incubated in the dark at 25°C. After one week sterile roots were

transferred onto fresh agar medium and any contaminated roots were discarded.

Any fungi which grew from the ectomycorrhizal roots after at least 2 weeks, were immediately sub-cultured onto BIA on which they were maintained, most of them being subcultured every 4 weeks. Stock cultures of all the isolates were kept on BIA slopes under mineral oil (liquid paraffin, S.G. 0·830-0·870).

The root isolates kept in culture are shown below (Table 2).

Table 2.

The Root Isolates.

Locality of ectomycorrhizal roots	Date of collection of ectomycorrhizal roots	Surface sterilant of ectomycorrhizal roots	Root isolate number
Brandon Forest	Autumn 1972	100 vols hydrogen peroxide	M1 to M6
Brandon Forest	Spring 1974	1% sodium hypochlorite	M31 to M33
Brandon Forest	Spring 1974	1% sodium hypochlorite	M65 to M78
Brandon Forest	Spring 1974	1% sodium hypochlorite	M80
Brandon Forest	Spring 1974	1% sodium hypochlorite	M88 to M91
Angley Woods	Autumn 1972	100 vols hydrogen peroxide	M7 to M30
Angley Woods	Spring 1974	1% sodium hypochlorite	M34 to M64
Angley Woods	Spring 1974	1% sodium hypochlorite	M79
Angley Woods	Spring 1974	1% sodium hypochlorite	M81 to M87

Young sporophores of proven ectomycorrhizal fungi, collected from Brandon Forest (Boletus variegatus 1, Boletus badius) and Angley Woods (Boletus variegatus 2, Boletus bovinus, Boletus luteus, Amanita rubescens), were also used to obtain cultures. Tissue was aseptically removed from the pileus, just above the stipe and was transferred to an agar plate of sterile MEA or BIA. Plates were then incubated at 25°C, and as soon as hyphae began to grow from the sporophore tissue, they were subcultured and maintained on BIA, with subculturing every 4 weeks. Cultures were also kept on BIA slopes under mineral oil.

In order to determine whether 100 vols hydrogen peroxide had any lasting adverse effect on the fungi isolated from the ectomycorrhizal roots, an experiment was carried out to determine the effect of various concentrations of hydrogen peroxide on the growth of M13 (a root isolate) and Boletus variegatus 1 (a sporophore isolate).

Four cultures of each, grown on BIA for 3 weeks, were flooded with solutions of 50 vols, 25 vols and 12·5 vols hydrogen peroxide. The cultures were then left for 30 minutes, the hydrogen peroxide was removed with a sterile pasteur pipette, and 3 mycelial plugs (5mm in diameter) were transferred from each culture to fresh BIA.

These cultures together with controls, were then incubated at 25°C, and their growth was recorded as the radius of the mycelium after 12 days.

The mycelial morphology of all the isolates maintained on BIA was examined macroscopically as well as microscopically, and the ultrastructure of M1, M8, and M23 (root isolates) and Boletus luteus (sporophore isolate) was examined using the electron microscope.

ULTRASTRUCTURE OF M1, M8, M23 AND BOLETUS LUTEUS.

Erlenmeyer flasks (250ml), each containing 50ml of BLM (see page 52) were inoculated with 3 mycelial agar plugs (5mm diameter) from a BIA culture and left for 12 days in the dark at 25°C. Each colony was then washed with distilled water, put into a 25mm glass petri-dish and covered with 2% non-sterile water agar (Purified agar, Oxoid). When set, 5 pieces of agar of 1mm cube, containing the fungal tissue, were cut out and subjected to the following fixation and embedding treatment.

The tissue was fixed in 5% buffered gluteraldehyde for 2 hours, rinsed twice in M/15 phosphate buffer (pH 7·2), for one hour each time, and then left overnight in the buffer at 4°C. Next day the tissue was rinsed in buffer, left in 1% osmium tetroxide for 4 hours, and was then rinsed twice with distilled water. The fixed material was subjected to the following series of acetone solutions followed by propylene oxide:-

a)	30% acetone	15 minutes
b)	50% acetone	15 minutes
c)	70% acetone	15 minutes
d)	90% acetone	15 minutes
e)	100%acetone, two changes,	20 minutes each
f)	50:50 propylene oxide:acetone	10 minutes
g)	100% propylene oxide	15 minutes

Finally the tissue was infiltrated with an epoxy resin mixture made up as follows:-

Epoxy resin mixture.

Epicote 812 (TAAB Laboratories, England)	20g
DDSA hardener (Dodecenyl succinic anhydride)	10g
NMA hardener (Nadic methyl anhydride)	10g
BDMA accelerator (Benzyldimethylamine)	0•8g

Initially 10% epoxy resin mixture was added to the last propylene oxide treatment, and left on a rotator overnight. This was followed by a 50% epoxy resin mixture which was allowed to infiltrate for 4 hours. The specimens were removed to 100% resin, left overnight, and then placed in fresh 100% resin for one day. Finally they were suitably orientated in embedding moulds (which contained fresh epoxy resin mixture) and placed at 60°C for 24-48 hours.

Ultrathin sections of the mycelium were cut with a glass knife using a Cambridge-Huxley Ultra-Microtome Mark 2, and then mounted on copper grids, which were covered with a thin film of 0•2% formvar. The sections were double stained, initially in a saturated solution of uranyl acetate for 15 minutes. They were then removed, thoroughly washed with distilled water and dried on filter paper. Finally they were placed in Reynolds lead citrate solution (see below) for 5 minutes and then again washed and dried.

Reynolds lead citrate solution.

a)	Lead nitrate	1•33g	and dissolved together shaken for 1 min.
	Sodium citrate	1•76g	
	Distilled water	30ml	
b)	N NaOH	8ml	add to (a) above.
	Distilled water	to 50ml	

Analar reagents were used where available.

After staining, the sections on the grids were observed with an AEI Corinth 275 Transmission Electron Microscope.

SYNTHESIS OF ECTOMYCORRHIZAE.

1. Fortin's Method.

(Fortin, 1966)

Initially glass petri dishes were filled with 50ml of the following substrates (Table 3) and to these substrates, Fortin's mineral medium or distilled water was added as shown:-

Table 3.

Substrates and media added to petri dishes.

Number of dishes	Substrate	Medium used	Volume of medium (ml)
10	1. Perlite	Mineral	25
10	2. Perlite+peat (14:1)	Mineral	25
10	3. Peat	Mineral	25
10	4. Peat+humus (4:1)	Mineral	25
5	5. Humus	Mineral	20
5	6. Humus	Distilled water	20
5	7. Humus (non-sterile)	Mineral	20
5	8. Humus (non-sterile)	Distilled water	20
10	9. Sand+peat (4:1)	Mineral	25

<u>Fortin's mineral medium.</u>

<u>(Fortin, 1966)</u>

$Ca(NO_3)_2 \cdot 4H_2O$	71·95mg
$MgSO_4 \cdot 7H_2O$	20·47mg
KH_2PO_4	10·00mg
Ferric citrate solution (1%)	1·00ml
Trace element solution	1·00ml
Distilled water	to 1000ml

pH 4·5 (Adjusted with N HCl)

<u>Fortin's trace element solution.</u>

<u>(Fortin, 1966)</u>

KCl	3·728g
H_3BO_3	1·546g
$MnSO_4 \cdot 4H_2O$	0·845g
$ZnSO_4 \cdot 7H_2O$	0·575g
$CuSO_4 \cdot 5H_2O$	0·125g
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0·018g
Distilled water	to 1000ml

Analar reagents were used where available.

The mixture of perlite and peat (14:1) used, is that recommended by Marx and Zak (1965) with vermiculite and peat. It is claimed to maintain the medium constant at pH 4·6.

The humus was obtained from the litter layer under pines in Angley Woods, Kent, and was collected in April 1973. It was sieved through a wire sieve of pore diameter 5mm, to remove all the large particles.

Only 20ml of liquid media was added to the humus as it tended to be slightly damp. The perlite, peat/perlite and

sand/peat mixtures were all sterilized, in their petri dishes with the mineral medium added, at 15 p.s.i. for 15 minutes, whereas the peat, peat/humus and humus mixtures, again with the liquid already added, were sterilized by steaming for one hour on three consecutative days.

Seeds of *Pinus sylvestris* (seed number 69(4253)1) from Thetford Forest, were purchased from the Forestry Commission, Alice Holt Lodge, Wrecclesham, Farnham, Surrey. To germinate these seeds aseptically, they were first sterilized by immersing them in 100vols hydrogen peroxide with a drop of "Tween 80", for 30 minutes (Smith, 1973). This not only sterilized them, without injury to the seed, but also stimulated their germination (Trappe, 1961). The hydrogen peroxide was removed using a sterile Pasteur pipette and then the seeds were aseptically transferred to petri dishes containing sterile dilute MEA (0•5% malt extract).

Malt extract agar (0•5%).

Malt extract (Oxoid)	5g
*Oxoid agar no. 3	12g
Distilled water	to 1000ml

pH 5•5. Autoclaved at 15p.s.i. for 15 minutes.

* Later media contained Agar Agar (Solmedia).

The petri dishes were incubated, lid up, at 25°C in the dark. Germination after one week was about 80%.

Seedlings of *Pinus sylvestris*, grown for 30 days aseptically and with the average length of the root 25mm, were treated as follows. The root tip was aseptically removed and also most

of the hypocotyl, leaving about 5mm of the latter attached to the root. The hypocotyl of each explant was then pushed gently into organic medium contained in a glass vial

Fortin's organic medium.

(Fortin, 1966)

Sucrose	175·0g
Thiamin HCl	1·0mg
Choline chloride	2·5mg
Distilled water	to 1000ml
Purified agar (Oxoid)	10g

Analar reagents were used where available and the medium was autoclaved at 5 p.s.i. for 45 mins.

The glass vial was buried in the petri dish substrate, making sure that the root was completely buried. Inoculum consisted of fungal macerate (see (a) below) of one of the root isolates, M1, M3 or M7, and was added to the substrate in the region of the root, but at a distance from the vial, in an effort to prevent growth of the fungus into the organic medium.

All processes were carried out aseptically, each dish being sealed with masking tape, to prevent contamination and reduce evaporation. The dishes were incubated at 21°C for at least 8 weeks.

The above procedures were repeated with certain variations.

a) Preparation of fungal macerate.

In all the following variations, a fungal macerate was used as a source of inoculum and was prepared as follows.

Three mycelial agar plugs, cut from the edge of a mycelium of one of the isolates were grown, submerged, in 20ml of sterile BLM (see page 52) contained in a 100ml Erlenmeyer flask with a layer of 5mm glass beads on the bottom of the flask. The cultures were incubated at 25°C in the dark for 10 days, and then the mycelia were macerated by hand shaking the flasks for 2 minutes. Three drops of this macerate were used to inoculate each Fortin petri dish.

b) Filtered fungal macerate as inoculum.

Fungal macerates of two root isolates, M8 and M16 were prepared (as in (a) above), but the macerate was first passed through a fine dental gauze before use (see Fig. 1). The same substrates and media were used as before.

c) Use of sterile lanolin to seal the glass vials.

The above procedures were repeated (using the fungal macerate as in (a) above) with M8 and substrates 3, 4, 5 and 7 (see Table 3, page 61) but the vials containing the organic medium were sealed around the inserted hypocotyls with sterile lanolin (sterilized at 105° for approximately

Fig. 1. Apparatus for preparing sieved mycelial suspensions.

16 hours, Lundeberg 1960). The lanolin was left to set before the vials were aseptically buried in the substrate.

d) Inoculation of petri dishes with a "massive" dose of non-filtered fungal macerate.

The following isolates were used as a source of inoculum, and were added to petri dishes which contained perlite plus 15ml of Fortin's mineral medium (neat or diluted 1 to 4 with water). Isolates M2, M4, M9, M10, M11, M13, M15, M21, M23, M28, M32, M37, M44B, M48, M65, M78, *Boletus variegatus* 1 and *Boletus luteus* were used.

The fungal macerate was prepared as on page 65 (a) except that 10 colonies, grown in BLM, were transferred to bead flasks containing 10ml of Fortin's mineral medium (or diluted mineral medium) and then the contents of each flask (except the glass beads) were poured into each petri dish after maceration. Also the colonies of *Boletus variegatus* 1 and *Boletus luteus* were grown floating on the surface of the BLM, the latter being grown for 17 days in BLM, instead of 10 days.

e) Non-sterile humus as a substrate.

In order to determine whether ectomycorrhizae would form in non-sterile soil after inoculation with a root isolate and the addition of a fungicide (which slows down the growth of soil fungi but

which does not effect Basidiomycetes, Taylor 1971), the above procedures were repeated using humus collected from Angley Woods, Kent. In this experiment only the mineral medium and Benomyl (a selective fungicide) were sterilized, 25ml of mineral medium being added to the humus with Benomyl at a concentration of 10ppm. The isolate used in this experiment was M23.

f) Inoculation of petri dishes with two root isolates.

To determine the effect of inoculating 2 fungal isolates into one petri dish, the following combinations (chosen at random) were used. Each combination was replicated 10 times, using perlite and peat plus perlite (1:14) as substrates with Fortin's mineral medium. The combinations used were M8 + M16 and M1 + M3.

2. Pachlewski's Method.

(Pachlewski, 1967; Pachlewska, 1967; Pachlewski and Pachlewska 1967)

Ten boiling tubes for each fungal isolate were prepared containing 50ml of Pachlewski's starvation medium (Pachlewska 1967) as shown below:-

Pachlewski's Starvation Medium.

Thiamin HCl	100ug
Purified agar (Oxoid)	15g
Distilled water	to 1000ml

pH 5•5–6•0

They were autoclaved at 15p.s.i. for 15 minutes and then cooled at an angle, so that the agar in the tubes formed a shallow slope. Seeds of Pinus sylvestris were sterilized and germinated as described on page 63 the period for germination being 10 days.

One Pinus sylvestris seedling of radicle length approximately 10mm was aseptically transferred to each agar slope, so that the radicle grew downwards over the agar surface. They were then subjected to the following treatments:-

a) Tubes inoculated with root isolates after 4 weeks seedling growth on agar medium.

Five tubes were wrapped in aluminium foil and were left at room temperature, to allow the root to grow into the substrate before inoculation. After 4weeks, two mycelial agar plugs (see page 58) were transferred aseptically into each tube, the plugs being placed on the agar surface near to the root. The tubes were then transferred to racks in the greenhouse which was maintained at a temperature of 20°C during the winter, but varied in the summer from 15 to 30°C. The light intensity at plant level was equal to 8036 lux.

b) <u>Tubes inoculated immediately with root isolates.</u>

The remaining 5 tubes were inoculated immediately with two mycelial agar plugs and after wrapping them in aluminium foil, they were placed in racks and incubated in the greenhouse as before.

The above methods and treatments were used for a range of root and sporophore isolates.

Six months after the tubes had been prepared, a further inoculum (5ml) of fungal macerate was added to each tube. This was prepared as on page 65(a) , except that colonies grown in BLM were transferred to bead flasks containing sterile distilled water at the rate of 1ml water per colony. The macerate was therefore prepared in water. (This procedure was not repeated for M12 as it does not grow successfully in liquid culture).

In a further experiment with some isolates, an initial inoculum of 5ml of fungal macerate, prepared as above, was used. No further inoculum was added.

3. <u>Melin's Method.</u>

This method was first used by Melin (1921) and later modified by Norkrans (1949), Hacskaylo (1953) and Ferry (1967). Also the vermiculite was replaced by perlite as a substrate. Two litre Erlenmeyer flasks were used and filled with one litre of perlite, also to each flask, 600ml of the following nutrient solution was added:-

Melin-Norkrans nutrient solution.

(Norkrans, 1949)

KH_2PO_4	0•5g
$CaCl_2 \cdot 6H_2O$	0•1g
NaCl	0•025g
$MgSO_4 \cdot 7H_2O$	0•15g
$(NH_4)_2HPO_4$	0•25g
Fe-citrate (1%)	1•2ml
Glucose	2•5g
Thiamin HCl	0•015mg
Distilled water	to 1000ml

pH after autoclaving = 5•0

Analar reagents were used where available and the flasks were autoclaved at 15 p.s.i. for 30 minutes.

In some cases one litre of a mixture of perlite and peat (840:60) was used in each flask, the peat being added to maintain the pH in the flask after autoclaving at pH 4•6, claimed to be a suitable pH for ectomycorrhizal formation (Marx and Zak, 1965).

Seeds of *Pinus sylvestris* were sterilized and germinated (see page 63) and after one week, three of the seedlings were transferred aseptically to each flask and left at room temperature for 14 days. Three flasks of perlite and three flasks of the peat/perlite mixture were then inoculated with 10ml of filtered fungal macerate (prepared as on page 70 and page 65(b)) of each isolate tested.

All flasks were left in the greenhouse,

at a temperature of 20°C in the winter, but which varied in the summer between 15°C to 30°C. The light intensity was equal to 8036 lux at plant level.

After 3 months, one flask of each isolate and treatment was opened, the roots washed free of substrate and the seedlings examined for any ectomycorrhizal development. After 7 months the remaining flasks were re - inoculated with a massive dose of fungal macerate. This was prepared as before, except that the colonies were macerated in sterile Melin - Norkrans nutrient solution (page 71), instead of distilled water, and also the macerate was not filtered. Twenty - five millilitres of macerate was added to each flask. These seedlings were examined after 26 months growth.

4. Controlled Environment Method.

In order to maintain the flasks in a more controlled environment e.g. temperature, further modifications of Melin's method were developed.

One litre of perlite was again added to each flask of 2 litre capacity. In the top of the flask a glass tube (2cm diameter and 50cm long), was inserted until it just touched the surface of the perlite and was wedged in the neck of the flask with non - absorbent cotton wool. Melin - Norkrans nutrient solution (400ml), as used in the previous method, was added to each flask, the Fe - citrate solution being replaced by 6·0mg/litre of ethylamine - diaminetetra - acetic acid (Fe salt) i.e. Fe - EDTA (Smith, 1973). Flasks of diluted nutrient solution (diluted 1 to 3 with

distilled water) were also prepared. The glass tubes were then plugged with cotton wool and autoclaved at 15 p.s.i. for 30 minutes.

The unfiltered fungal macerate was prepared as on page 72 i.e. in nutrient or diluted nutrient solution. Twenty - five millilitres of macerate was added to 175ml of sterile Melin - Norkrans nutrient solution before being poured aseptically into the flasks via the glass tubes, the latter being used to stir the macerate into the substrate.

Ten germinated pine seedlings (see page 63), approximately 3 weeks old, were then transferred aseptically to each flask through the glass tube.

All flasks were left at room temperature for 3 weeks in order for the seedlings to establish themselves in the substrate, before they were transferred to a refrigerated water bath maintained at 20°C. The plants were also subjected to a 16 hour photoperiod and a light intensity at plant level of 18,750 lux.

The seedlings were removed for examination after 10 - 11 months.

5. Pot Experiments.

(Modification of Theodorou and Bowen, 1970)

Humus collected from Angley Woods, Kent, or Brandon Forest, Norfolk, was put through a sieve of pore size 5mm to remove all the larger fragments. Approximately 800ml of non - sterile humus was then put into each pot.

A fungal macerate was prepared (see page 70) and

25ml was thoroughly mixed with the humus using a sterile glass rod. Root isolates obtained from Angley Woods were inoculated into Angley humus and similary root isolates from Thetford were inoculated into Thetford humus. Ten pine seedlings which had been aseptically grown for 3 weeks (see page 63) were then added to each pot. The pots were left in the greenhouse at 20°C in winter, 15 - 30°C in summer with an average light intensity at plant level equal to 8036 lux.

A further experiment was set up using sterile humus (autoclaved for 2 hours at 15 p.s.i., Theodorou and Bowen, 1970) contained in pots which had been surfaced sterilized with alcohol and ultra - violet light for one hour. Finally a layer of sterile sand was placed on the humus after the pots had been inoculated.

All pots were watered when necessary with sterile distilled water, initially from the base of the pot, but later, on the humus/sand surface. The seedlings were removed from all pots 8 months after inoculation.

THE EFFECT OF INDOLE - 3 - ACETIC ACID (IAA) ON THE DEVELOPMENT OF PINUS SYLVESTRIS ROOT SYSTEMS.

In order to compare the effects of fungal isolates and IAA on pine seedlings, the following experiment was undertaken.

Tubes of Pachlewski's starvation medium were prepared as before (see page 69), a 10 day old aseptically germinated pine seedling being added to each tube.

IAA solution (sterilized by micropore filtration) was added aseptically to each tube to give final concentrations ranging from 10^{-4} to 10^{-11} M. The IAA was added 3 times per week, in order to maintain the IAA content in each tube.

The bottom of the boiling tubes up to the surface of the agar were wrapped in aluminium foil and they were then left in the greenhouse which was at a constant temperature of 20°C in the winter, but which varied between 15 and 30°C in the summer. The average light intensity was 8036 lux. Observations on the formation of the lateral roots were made over the course of 9 months.

In a further experiment IAA was not added until 4 weeks after pine seedlings were placed in the tubes.

NUTRITIONAL EXPERIMENTS.

1. Preparation of experimental media.

The basic liquid medium (BLM) and basic ion agar (BIA) which were used in these experiments are modifications of those used by Melin (Melin et al., 1954) and are described on page 52.

The maximum liquid medium (MLM) and maximum ion agar (MIA) consisted of the basic media with the following amino acids and vitamins also added per litre.

Amino acids.

Amino acid	Amount
Glycine	2•5mg
DL - - Alanine	9•5mg
DL - Valine	39•5mg
L - Leucine	24•5mg
DL - Isoleucine	24•5mg
DL - Phenylalanine	19•5mg
L - Tyrosine	33•0mg
L - Tryptophan	11•0mg
L - Glutamic acid	161•0mg
L - Aspartic acid	20•5mg
L - Proline	45•5mg
L - Hydroxyproline	1•0mg
DL - Serine	2•5mg
DL - Threonine	17•5mg
L - Cystine	1•5mg
DL - Methionine	17•0mg
L - Arginine	19•0mg
L - Histidine HCl	12•5mg
L - Lysine HCl	30•0mg

Vitamins.

Riboflavin	0•05mg
Ca-pantothenate	0•05mg
Folic acid	0•05mg
Niacin	0•05mg
Para-aminobenzoic acid	0•05mg
Pyridoxine HCl	0•05mg
Biotin	0•0005mg
Choline chloride	0•5mg
Inositol	50•0mg

Analar reagents were used where available.

The amino acid and vitamin mixtures were always sterilized separately and were added aseptically to other medium constituents to produce MLM or MIA (pH 4•6).

Usually 50ml of liquid medium was used in 250ml Erlenmeyer flasks, and approximately 30ml ion agar in 9cm petri dishes.

2. Preparation of inocula for experiments.

Mycelial agar plugs, 5mm in diameter and cut from the edge of growing colonies were used for agar plate experiments, after being leached in sterile distilled water. Fungal macerate was used for liquid media experiments. This latter inoculum was prepared in sterile distilled water (page 70) and unless otherwise stated was always filtered (see page 65 (b)).

3. Growth assessment.

For those experiments which were performed in liquid culture, growth was assessed by dry weighting the mycelia at 70°C. When roots were used in experiments they were separated from the fungus before the mycelium was dried.

When solid agar medium was used in an experiment, the radius of the colony was measured to the nearest millimetre and always along the same line of growth.

In these experiments, the treatments were replicated three times and incubation was at 25°C, unless otherwise stated.

Standard errors are given with the means. The difference between two means (one being the control, unless otherwise stated) is considered significant when the value of $P = 0{\cdot}05$ or less, as determined by the "Students" t - distribution (unless stated otherwise). When no significance occurred, this was indicated by the abreviation "n.s.".

4. Growth in BLM and MLM and in mineral solution.

The growth of selected root isolates was measured in BLM and in MLM, using 0•5ml of filtered fungal macerate (see pages 70 and 65(b)). In two cases (M1 and M3) growth curves over a period of 26 days were obtained both in liquid media and on agar media.

All subsequent nutritional experiments were conducted with M13, mainly because this isolate shows a strong positive response to roots (see page 175 of results section) and is a reasonably fast growing isolate. Also 0•5ml of filtered fungal macerate was used per flask of liquid medium (see pages 70 and 65(b). The response of M13 to various components of MLM was

measured, including thiamin, the amino acid mixture and the vitamin mixture.

The effect of temperature and pH on the growth of M13 were determined on MIA and in MLM respectively. The pH was controlled using the following buffers:-

Table 4.

The buffers added to MLM.

a) Sorensen's phosphate buffers.

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

b) Citric-acid-sodium citrate buffers(diluted from 0•1M to 0•01M).

pH	0•01M citric acid	0•01M Na_3citrate
3•0	18•6ml	1•4ml
3•6	14•9ml	5•1ml
4•0	13•1ml	6•9ml
4•6	10•3ml	9•7ml
5•0	8•2ml	11•8ml

Table 4. (cont.)

c) Phthalate - HCl buffers(0•05M diluted to 0•01M).

pH	0•2M KH phthalate	0•2N HCl	Dilutions
2•2	5ml	4•67ml	Diluted to 100ml with H_2O
2•6	5ml	3•295ml	Diluted to 100ml with H_2O
3•0	5ml	2•032ml	Diluted to 100ml with H_2O
3•6	5ml	0•597ml	Diluted to 100ml with H_2O
4•0	5ml	0•04ml of 0•2N NaOH	Diluted to 100ml with H_2O

In all cases the buffers were autoclaved seperately, and then 9ml of buffer was incorporated into 39ml of sterile MLM in 250ml Erlenmeyer flasks.

The ability of M13 to utilize various nitrogen sources, in BLM, was determined, the nitrogen sources being ammonium tartrate, potassium nitrate and peptone. All were used at a concentration of 0•0027M nitrogen and were sterilized (by autoclaving) seperately from the rest of the medium.

The effect of the following carbon sources in BLM, on the growth of M13 was obtained. These were sterilized by steaming with the exception of fructose and xylose which were sterilized by micropore filtration.

Table 5.

The carbon sources added to BLM.

C - source added to give a final concentration of 2%. (Analar reagents used where possible)	pH adjustment to give final pH 5•0 (per 400ml of BLM)
Glucose	-
Fructose	-
Galactose	-
Mannose	-
Xylose	0•3ml N/1 NaOH
Mannitol	-
Maltose	-
Sucrose	-
Sodium acetate	10ml N/1 HCl
Sodium malate	5ml N/1 HCl

The ability of M13 to utilize various polysaccharides was also tested in BLM. The following polysaccharides were used.

Table 6.

The polysaccharides added to BLM.

Polysaccharide and concentration	Origin	pH adjustment to give a final pH of 5•0
2% Starch	B.D.H.	-
1% Pectin	Sunkist Growers	2ml N/1 NaOH per 400ml
1% Sodium polypectate	Sunkist Growers	-
1% Carboxymethyl cellulose (CMC)	Hercules	1ml N/1 HCl per 400ml

In addition the effect of these polysaccharides with the addition of 0•05% glucose was determined.

The ability of selected root isolates, M13, M23, M37 and M65, to utilize the cellulose and lignin present in the sawdust of Pinus sylvestris wood was determined.

Fahraeus' method was used with modifications (Fahraeus et al., 1949). Five grams of air - dried sawdust (equal to 4•8g of oven-dried sawdust), which had been passed through a sieve of pore size 2mm, was transferred to a 250ml Erlenmeyer flask, with 40ml of the following solution.

<u>Mineral solution.</u>

<u>(Fahraeus et al., 1949)</u>

KH_2PO_4	0•5g
$MgSO_4 \cdot 7H_2O$	0•5g
$FeSO_4 \cdot 7H_2O$	0•01g
Distilled water	to 1000ml

pH after autoclaving = pH 5•3

Analar reagents were used where available.

Six flasks were prepared for each root isolate, 0•1% glucose being added to three of these flasks. They were then autoclaved at 15p.s.i. for 30 minutes. After cooling, the flasks were inoculated with 0•5ml of filtered fungal macerate (see page 70 and page 65(b)) and were then incubated at 25°C in the dark for six months. Sterile distilled water was added after 8 weeks to compensate for the loss of water by evaporation, the amount being determined by weighing the control flasks.

At the end of the experimental period, the contents of the flasks were dried to a constant (dry) weight at 65°C for 3 days.

After 3 days equilibration at normal air humidity the contents of replicate flasks were pooled, ground in a pestle and mortar, and were then analysed for lignin and cellulose as follows:-

a) Determination of lignin.

(Bengtsson, 1925, 1938; Lindeberg, 1944, 1946; Fahraeus et al., 1949; Norkrans, 1950; Brauns, 1952)

The method used for the determination of lignin was similar to that used by Brauns (1952). Samples of approximately 350mg dry weight (see page 166 of results section for actual dry weight), were digested, at 100°C, with 3ml of thioglycolic acid (Sigma grade II) dissolved in 40ml 2N HCl, for 4 hours with occassional shaking. The digest was then cooled, filtered through a Buchner funnel containing Whatman no. 1 filter paper, and then the residue was washed with 100ml distilled water and air dried.

150ml N/15 KOH was added to this residue in a stoppered 250ml bottle and shaken for 24 hours, the lignin - thioglycolic acid complex entering into solution. The remaining residue was again filtered off, this being kept to one side for the cellulose analysis, after washing it with approximately 100ml distilled water. To the filtrate, 20ml of 5N HCl was added to precipitate the lignin - thioglycolic acid complex, which was then left standing overnight for the brownish precipitate to settle out.

The precipitate was filtered through a Whatman no. 40 filter paper in a Buchner funnel and then washed with distilled water. Finally it was dried at 80°C until a constant dry weight was obtained, the quantity of lignin in the sample being

expressed as lignin - thioglycolic acid complex.

Two hundred, four hundred, six hundred and eight hundred milligram fresh weight samples of sawdust were used in order to obtain a correction curve for the fraction of lignin - thioglycolic acid complex which does not precipitate out with 5N HCl (Lindeberg, 1946).

b) Determination of cellulose.
(Lindeberg, 1946; Fahraeus et al., 1949; Norkrans, 1950)

Thirty millilitres of freshly prepared Schweizer's reagent (cuprammonium hydroxide), prepared as below, was added to the washed residue insoluble in N/15 KOH, from the lignin determination (see above). This was then shaken in a screw cap bottle for one hour to dissolve the cellulose and the cellulose was precipitated out by the addition of 50ml 80% ethanol. The precipitate was left overnight to settle, and finally it was filtered through a Whatman no. 40 filter paper contained in a Buchner funnel, washed and then dried to a constant dry weight at 80°C.

i) Preparation of Schweizer's reagent.
(Brauns, 1952)

To 62•5g of cupric sulphate dissolved in 625ml water, 200ml of 2N NaOH (carbonate free) was slowly added, with vigorous stirring, until

no more cupric hydroxide precipitated from the solution. The supernatant was removed by filtration and then the precipitate was washed with distilled water until free of sulphate ions (tested with $BaCl_2$). This precipitate of cupric hydroxide, was transferred to a one litre bottle, without being dried and finally just dissolved in 0•88 ammonia to form the deep blue, saturated solution, of cuprammonium hydroxide.

The growth of the same four isolates on filter paper (cellulose) incorporated into BIA was determined. Filter paper which had been ground to a pulp with a little water, was substituted for the glucose in BIA at the rate of 20g per litre. The medium was autoclaved at 15 p.s.i. for 15 minutes.

EXPERIMENTS WITH EXCISED TOMATO ROOTS.

Culture of excised tomato roots.

Seeds of tomato of the variety Alsa Craig were used, and were sterilized and germinated as for the culture of pine seedlings (see page 63). One germinated seed was selected for culture. Seven days after germination, the radicle tip was removed and the root severed from the hypocotyl. The excised root then being transferred to a 250ml Erlenmeyer flask containing 50ml sterilized modified White's medium (Ferry, 1967).

Modified White's medium.

a) Carbon source.	Sucrose	20•0g
b) Macro-nutrients.	$Ca(NO_3)_2 \cdot 4H_2O$	0•2g
	$MgSO_4 \cdot 7H_2O$	0•36g
	KNO_3	0•08g
	KCl	0•065g
	Na_2SO_4	0•2g
	$NaH_2PO_4 \cdot 2H_2O$	0•0165g
c) Trace-nutrients.	KI	0•75mg
	$FeSO_4 \cdot 7H_2O$	2•5mg
	$MnSO_4 \cdot 4H_2O$	0•5mg
	$ZnSO_4 \cdot 7H_2O$	1•5mg
	H_3BO_3	1•5mg
d) Vitamins.	Thiamin HCl	0•1mg
	Niacin	0•5mg
	Pyridoxine HCl	0•1mg
e) Distilled water.	to	1000ml

Analar reagents were used where available and the medium was autoclaved at 15 p.s.i. for 15 minutes.

These roots (see Fig. 2), were then subcultured either every week (Clone A), or every 10 days (Clone B). The first clone (A) with a 7 day dry weight of 1•9mg, lost its vigour after 6 months, the second clone (B), with a 10 day dry weight of 6•2mg lasted for 12 months. To subculture the roots, the root tips were first severed from the laterals and then the laterals were severed from the main root. Those laterals bearing 5 to 8 secondary laterals were transferred to fresh White's medium.

Killed excised tomato roots were prepared from roots which had been grown in White's medium, either for 7 days (CloneA) or 10 days (Clone B). These were plunged into boiling water for 5 seconds and transferred to flasks to be used as required in the experiments.

1. Experiments with live and killed excised tomato roots.

a) Growth of various isolates in the presence of excised tomato roots in MLM.

50ml of MLM in a 250ml Erlenmeyer flask was inoculated with 0•5ml filtered fungal macerate (see pages 70 and65(b)), nine flasks being prepared for each fungus. To three flasks, one live excised tomato root was added, and to another three flasks one killed excised tomato root was added.The remaining three flasks were used as controls i.e. lacking roots. All flasks were incubated at 25°C in the dark for 7 or 14 days, when the fungus was separated from the root, filtered and dried (see page 78 for details).

Fig. 2. Ten day old excised tomato root (Clone B).

The following fungal isolates were used with Clone A roots:-

M1, M2, M3, M4, M5, M6, M7, M8, M9, M10, M11, M13, M15, M16, M21, M22, M23, and Boletus variegatus 1.

Clone B roots were used with the following root isolates:-

M13, M37, M44B, M48, M65, M70, and M78.

In all subsequent experiments Clone B roots. were used.

b) Seventeen - day time course experiment with M13 in MLM.

The effect of 10 day old live and killed excised tomato roots (Clone B) on the growth of M13 in MLM over a period of 17 days was further bb - served.

c) Growth of M13 and M23 in the presence of excised tomato roots in BLM, and MLM for 14 days.

The 14 day experiment was repeated for M13 and M23 (see section (a) above) but using both BLM and MLM and roots of CloneB.

d) Growth of M13 (continuous laboratory culture and culture maintained under oil) in the presence of excised tomato roots in MLM.

It is known that over a period of time the growth requirements of a continually subcultured fungus can change (How, 1940). An experiment to

determine the effect of excised tomato roots (Clone B) on the growth of M13 which has continuously been subcultured for two years in the laboratory, and M13 which had been kept under oil for two years was prepared. Flasks were inoculated with 0•5ml filtered fungal macerate (see pages 70 and 65(b)) were incubated at 25°C in the dark for 14 days.

e) <u>Growth of M13 in the presence of a cotton "root" and an extracted excised tomato root, both with and without live excised tomato root exudate (micropore filtered).</u>

In order to determine whether these fungi were stimulated only by the presence of supporting strands in the medium, and not by any substance exuded by the roots, the growth of M13 in the presence of a cotton "root" was observed.

Cotton "roots" were prepared by tying four strands of white cotton thread, 21cm long, together in the middle and then leaching them overnight in distilled water. One cotton "root" was placed separately in 250ml Erlenmeyer flasks, each containing 50ml distilled water and autoclaved at 15 p.s.i. for 15 minutes. These "roots" were then aseptically transferred to 50ml sterile MLM, containing 0•5ml filtered fungal macerate (see pages 70 and 65(b)), in 250ml Erlenmeyer flasks. The flasks were then incubated at 25°C for 14 days in the dark.

The effect of a cotton"root" together with excised tomato root exudate on the growth of M13 in MLM was determined. Micropore filtered live excised tomato root exudate (the equivalent of 6•2mg dry weight of excised tomato root, prepared as on page 93), was added to all flasks, except three control flasks. The flasks were then inoculated and incubated as before, for 14 days.

The growth of M13 in the presence of an excised tomato root which had previously been extracted with boiling water was also determined. Roots were boiled in distilled water for 20 minutes and then transferred aseptically to 250ml flasks containing sterile distilled water, in which they were leached for approximately 8 hours. They were finally transferred to flasks containing 50ml sterile MLM. These flasks were then inoculated and incubated as above, for 14 days.

The effect of micropore filtered live excised tomato root exudate together with an extracted root on the growth of M13, was also determined following the same procedure as with the cotton"roots" (see above).

f) <u>The effect of enclosing excised tomato roots within dialysis membrane bags on the growth of M13 in MLM.</u>

A final experiment was conducted with roots. Live and killed excised tomato roots (Clone B)

were aseptically enclosed in sterilized (autoclaved) dialysis membrane bags containing 8ml FLM each. These bags were then transferred into 250ml flasks each containing 50ml FLM and the flasks were inoculated with 0•5ml filtered fungal macerate of M13 (see pages 70 and 65(b)). Controls were prepared including dialysis membrane bags containing no roots.

g) <u>The effect of autoclaving dialysis membrane bags.</u>

In order to determine whether autoclaving alters the structure of dialysis membrane bags, the ability of both autoclaved and non-autoclaved dialysis membrane bags to dialyse a starch/ammonium sulphate mixture was tested. The bags were left suspended in 250ml distilled water for 4 hours, when the dialysate was tested for ammonium sulphate (with 0•05M $BaCl_2$) and starch (with iodine).

2. Experiments with excised tomato root exudate, extract and homogenate.

Preparation of exudate, extract and homogenate of excised tomato roots.

These root preparations were prepared as follows using Clone B excised tomato roots.

The exudate of killed or live excised tomato roots was collected from roots which had been grown for 10 days in White's medium (see page 87). The roots were transferred either immediately or after killing them, to

50ml sterile distilled water contained in 250ml Erlenmeyer flasks. They were left in the dark at 25°C for 14 days and were then removed from the flasks. The root exudate was concentrated under vacuum on a rotary evaporator at 40°C to give 6•2mg dry weight of excised tomato root (equivalent to one excised tomato root) per 0•5ml solution.

Excised tomato root extract was prepared by placing 10 day old live excised tomato roots in boiling distilled water for 20 minutes and then concentrating the extract as above. The extract was finally micropore filtered to remove any debris.

Homogenate was also prepared using 10 day old live excised tomato roots. They were placed in a Waring blender, covered with distilled water and macerated for 2 minutes. The debris was removed by filtering through a Buchner funnel containing Whatman no. 1 filter paper and finally the filtrate was concentrated as above. Any remaining debris was removed by micropore filtration.

a) Growth of M13 in the presence of excised tomato root exudate, from live and killed roots (collected in distilled water), extract and homogenate in MLM.

The growth of M13 in the presence of micropore filtered and autoclaved excised tomato root exudate (live and killed), extract and homogenate was tested, the equivalent of 6•2mg dry weight of excised tomato root preparation (in 0•5ml) being added to each flask containing 50ml MLM and 0•5ml filtered fungal macerate (see pages 70 and 65(b)). The

flasks were then incubated at 25°C for 14 days.

b) Growth of M13 at two different levels of micropore filtered excised tomato root exudate from live and killed roots (collected in distilled water), extract and homogenate in MLM.

M13 grown at two different levels of micropore filtered excised tomato root exudate from live and killed roots, extract and homogenate was observed. The flasks were inoculated and incubated as above (section (a)).

c) Growth of M13 in the presence of excised tomato root exudate, collected daily (in distilled water) in MLM.

Further experiments were undertaken collecting the killed and live excised tomato root exudate daily for 14 days, after which time the samples were pooled and then evaporated as before, except that this root exudate was evaporated to give a final concentration of 6•2mg dry weight of excised tomato root equivalent to 1•0ml. Half of the samples were autoclaved the rest being micropore filtered.

A similar experiment was carried out as above, except that the exudate, collected and concentrated daily, was added to the flasks containing M13 on the same day as the exudate was collected. It was sterilized by micropore filtration or autoclaving. All the flasks were inoculated and incubated as in section (a).

d) Growth of M13 in the presence of dialysate and retentate of tomato root exudate (from live excised tomato roots, collected in distilled water), extract and homogenate in MLM.

The effect of dialysing excised tomato root exudate from live roots, extract and homogenate against water was determined. Up to 8ml of root exudate, concentrated to 6•2mg dry weight of root per 0•5ml, was dialysed against 250ml distilled water at 6°C for 3 hours, with three changes of the water during this time. The dialysate and retentate were then readjusted to 6•2mg dry weight of excised tomato root per 0•5ml medium. Half of each fraction was then autoclaved, and the remaining half micropore filtered. 0•5ml of dialysate or retentate was added to 250ml Erlenmeyer flasks containing 50ml MLM inoculated with 0•5ml filtered fungal macerate of M13 (see pages 70 and 65 (b)).

This procedure was repeated for extract and homogenate of excised tomato roots. All the flasks were incubated as in section (a).

e) <u>Growth of M13 in MLM, in the presence of dialysate and retentate collected in distilled water from live exuding excised tomato roots enclosed within dialysis membrane bags.</u>

An experiment was also conducted with live excised tomato roots enclosed aseptically in sterilized dialysis membrane bags containing 8ml distilled water in each. These bags were transferred to 250ml flasks containing 50ml sterile distilled water and left in the dark at 25°C for 14 days. The roots were then removed and the dialysate and retentate evaporated to give the equivalent of 6•2mg dry weight of live excised tomato root per 0•5ml of root preparation. Their effect on the growth of M13 was tested. 0•5ml of each root preparation was added to flasks of MLM containing 0•5ml filtered fungal macerate (see pages 70 and 65(b)). The flasks were then incubated as in section (a).

f) <u>Growth of M13 in MLM, in the presence of live excised tomato root exudate (collected in MLM).</u>

The effect of exudate, collected from live excised tomato roots immersed in MLM instead of water, was determined on the growth of M13. Live excised tomato roots were transferred to flasks of MLM and left for 14 days at 25°C. After this time the roots were aseptically removed, half of the flasks were autoclaved and finally all flasks were

inoculated with 0•5ml filtered fungal macerate of M13 and incubated as above (section (a)).

g) Growth of M13 in MLM, in the presence of dialysate and retentate collected in MLM from live exuding excised tomato roots enclosed within dialysis membrane bags.

The experiment in which root exudate was collected from inside and outside the dialysis membrane bags was repeated (see section (e) above), except that the collecting medium both inside and outside the bag, was MLM. There was also no evaporation of the dialysate or retentate. 0•5ml filtered fungal macerate of M13 (see pages 70 and 65 (b)) was added directly to each flask containing 50ml autoclaved or micropore filtered MLM containing either dialysate, retentate or just MLM (i.e. control flasks). The flasks were then incubated as in section (a).

3. The effect of excised tomato root exudate from live and killed roots (collected in distilled water), extract and homogenate on the growth of Boletus variegatus 1 in MLM.

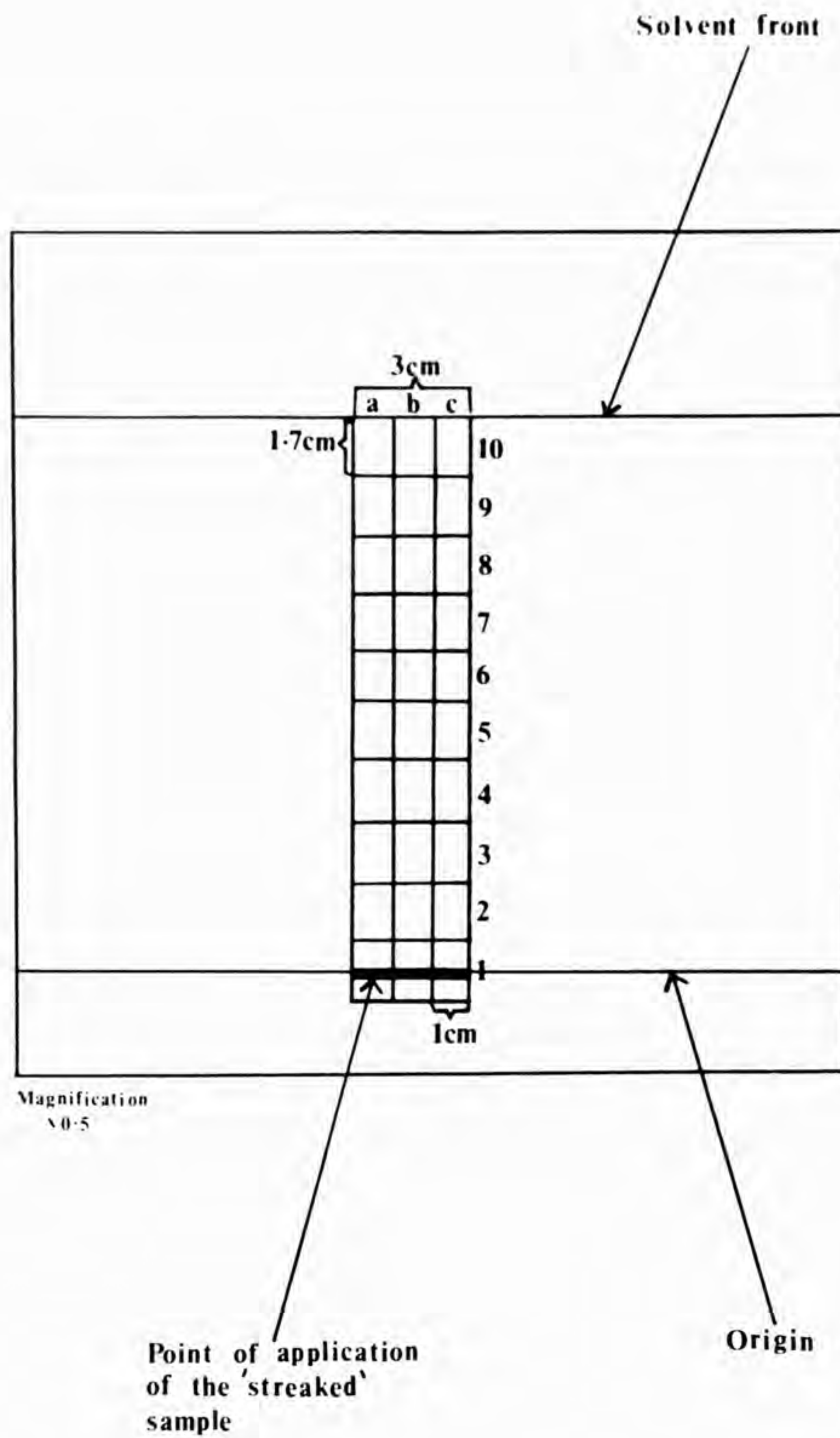
The effect of 0•5ml of excised tomato root exudate from live and killed roots, extract and homogenate was determined on the growth of *Boletus variegatus* 1 in MLM. The root preparations were either sterilized by micropore filtration or by autoclaving, and 0•5ml was added to each flask containing 0•5ml of *B. variegatus* 1 filtered fungal

macerate (see pages 70 and 65(b)), but passed through coarse dental gauze. Flasks were incubated at 25°C for 7 days.

4. Chromatographic bioassay of excised tomato root preparations.

1·5ml (equivalent of three excised tomato roots, see page 93) of excised tomato root exudate (from live roots collected in distilled water), extract or homogenate was loaded onto Whatman no. 1 Chromatography paper to form a streak 3cm wide at the origin. Fifty millilitres of butanol - acetic acid - water (BuA) solvent (60 : 15 : 25) or butanol - pyridine - water (BuP) solvent (60 : 60 : 60) (Smith, 1960) was placed in a Shandon Chromatography tank and allowed to stand for one hour before the chromatogram was run. The solvent was allowed to ascend for approximately 8 hours until the front was 16cm from the origin. The chromatogram was dried for 30 minutes and then cut into 10 fractions (see Fig. 3) from origin to solvent front, each fraction being divided into three portions, each being equivalent, therefore, to a loading of one root. The fractions were labelled 1 to 10 from origin to solvent front.

These fractions were sterilized under the ultra - violet (UV) light for one hour, being turned over after 30 minutes, in order to sterilize both sides of the paper. They were then transferred aseptically to flasks of sterile MLM containing 0·5ml filtered fungal macerate of M13 (see pages 70 and 65(b)) or *Boletus variegatus* 1



Magnification
x 0·5

Fig. 3. Preparation of the chromatogram for the bioassay of excised tomato root preparations.

(see page 98). Control flasks contained solvent treated fractions of chromatography paper. M13 flasks were incubated for 14 days and B. variegatus 1 flasks for 7 days at 25°C.

This procedure was repeated, except that the chromatogram fractions were sterilized by autoclaving in the flasks of MLM.

Additionally the effect of excised tomato root dialysate from live exuding roots (see page 97 section (e)) fractionated in BuA and sterilized by UV light was determined on the growth of M13.

EXPERIMENTS WITH PINE SEEDLING ROOTS.

Preparation of pine seedling root exudates, extracts and homogenates.

Pine seedling root exudate was collected over 25 weeks in growth tubes as seen in Fig. 4. These tubes consisted essentially of two parts. The upper half was an open ended glass growth tube in the base of which a rubber bung, covered by two layers of fine dental gauze was inserted. A thin glass tube was passed through a hole in the centre of the bung. The growth tube itself contained 15ml of fine acid washed silver sand, to which had been added 5ml of distilled water. Inserted in the cotton wool plug at the top of the growth tube was a pasteur pipette which had been plugged with cotton wool, the tip of the pipette just touching the surface of the sand. The bottom half consisted of a 100ml Erlenmeyer flask, plugged with cotton wool, through which the thin glass tube passing through the rubber bung was inserted. The Erlenmeyer flask was autoclaved separately from the upper growth tube with a spare glass rod inserted in the cotton wool plug. Following autoclaving the spare glass rod was removed and the whole apparatus assembled. (The apparatus was autoclaved at 15 p.s.i. for 30 minutes).

A 3 week old seedling of Pinus sylvestris (see page 63) was then aseptically transferred to each tube. The base of each tube was wrapped in aluminium foil and all tubes were incubated in the greenhouse at 20°C, where the light intensity was equal to 8036 lux.

Each week 10ml sterile distilled water was added through the pasteur pipette and the exudate collected in the Erlenmeyer flask.

After 25 weeks the exudate was pooled and finally concentrated

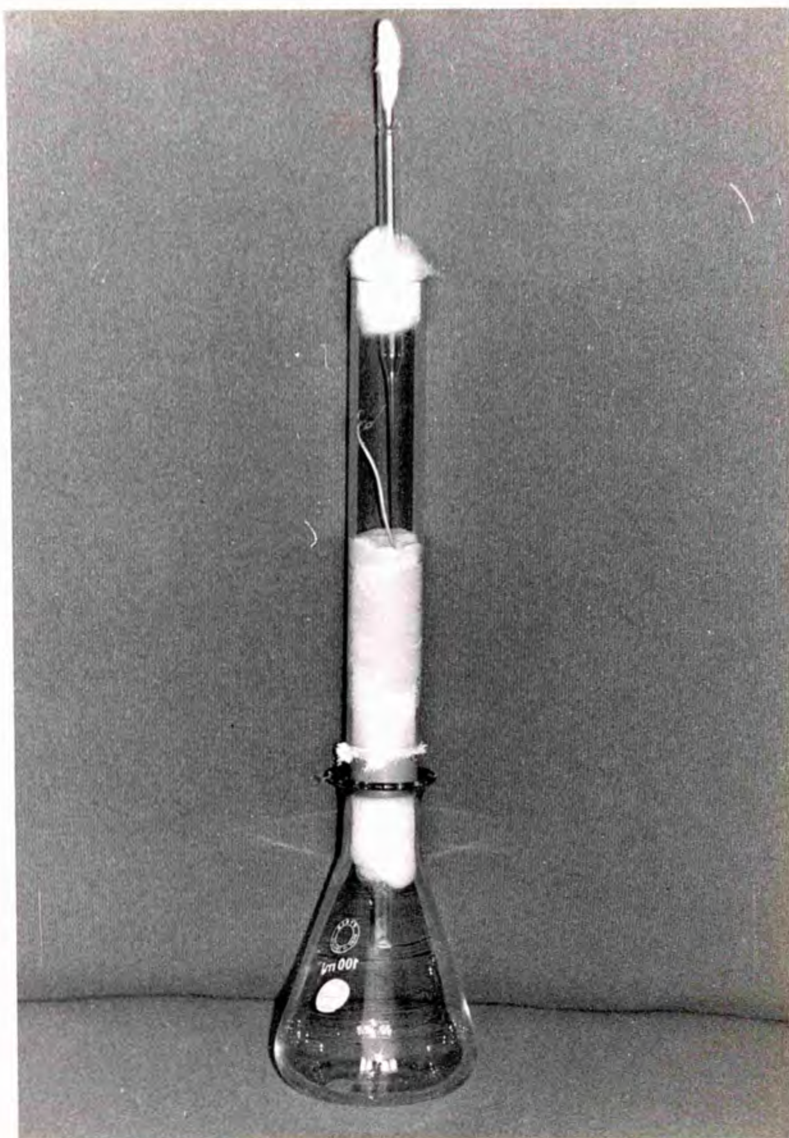


Fig. 4. Growth tubes for the culture of Pinus sylvestris seedlings. (Exudate collected for 25 weeks).

under vacuum on the rotary evaporator at 40°C to 6•2mg dry weight of pine seedling root per 0•5ml of exudate.

Also after removal of the pine seedlings from the tubes, half of the roots of the seedlings were extracted in boiling distilled water, and the other half homogenized (for the procedure of extraction and homogenization see the method used for the excised tomato roots, page 93). These samples were also evaporated to give a final concentration of 6•2mg dry weight of pine seedling root per 0•5ml of root preparation.

Pine seedling root exudate was also collected over 3 weeks using a modification of the apparatus used by Ferry (1967) for the collection of tomato seedling root exudate. Growth tubes were prepared as in Fig. 5. They consisted of a boiling tube, inside which a 4"x1" specimen tube was placed. Over the open end of the specimen tube, coarse gauze was tied and then distilled water was added to this tube to the level of the gauze. Finally the neck of the boiling tube was plugged with cotton wool and the tube was sterilized at 15 p.s.i. for 15 minutes.

Four, 3 week old seedlings of *Pinus sylvestris* (see page 63) were aseptically transferred to each growth tube making sure that the radicles passed through the gauze into the water below. The tubes were wrapped in aluminium foil to the level of the gauze and left at room temperature. The level of water in the specimen tube was kept constant by the addition of more sterile water to the tubes when necessary. After 3 weeks the pine seedlings were removed. Half of the roots were extracted and half homogenized (as described on page 93 for excised tomato roots). The pine seedling root exudate, extract and homogenate were all evaporated, under vacuum, on the rotary evaporator

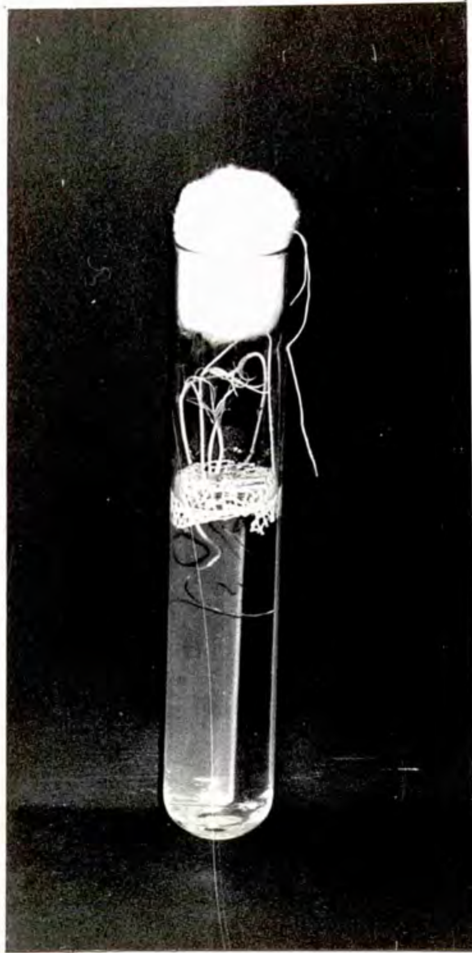


Fig. 5. Growth tubes for the culture of Pinus sylvestris seedlings. (Exudate collected for 3 weeks).

at 40°C to 6·2mg dry weight of pine seedling root per 0·5ml of root preparation.

1. <u>Experiments with pine seedling root exudates, extracts and homogenates in MLM.</u>

The effect of pine seedling root exudate (collected for 25 weeks) and extract and homogenate from these roots was determined on the growth of M13. The same method was used as for the excised tomato root extract, homogenate and exudate experiments, the pine seedling root preparations being either micropore filtered or autoclaved, and 0·5ml of pine seedling root preparation being added to each flask containing MLM with 0·5ml of filtered fungal macerate (see pages 70 and 65(b)) and incubated at 25°C for 14 days. This was repeated with 3 week pine seedling root preparations.

The effect of these pine seedling root preparations on the growth of <u>Boletus variegatus</u> 1 was also determined. Coarsely filtered fungal macerate of <u>B. variegatus</u> 1 (see page 98) was used as a source of inoculum with the 3 week pine seedling root preparations and the flasks were incubated for 7 days at 25°C. The only 25 week pine seedling root preparation tested on the growth of <u>B. variegatus</u> 1 was micropore filtered exudate, because of the lack of material. Also with this root preparation finely filtered <u>B. variegatus</u> 1 fungal macerate (see pages 70 and 65(b)) was used as a source of inoculum and the flasks were incubated at 25°C for 14 days.

2. Chromatographic bioassay of pine seedling root exudates in MLM.

The same procedure was used as for excised tomato root preparations except for *Boletus variegatus* 1 with pine seedling root exudate collected for 25 weeks, when finely filtered *B. variegatus* 1 fungal macerate (see pages 70 and 65(b)) was substituted for the coarsely filtered fungal macerate and the flasks were incubated for 14 days at 25°C. Also only one solvent system (BuA) was used and only pine seedling root exudate was assayed because of lack of material.

CHROMATOGRAPHY OF EXCISED TOMATO ROOT EXUDATE (FROM LIVE ROOTS COLLECTED IN DISTILLED WATER), EXTRACT AND HOMOGENATE.

The techniques used are those based on Smith (1960). Most of the chromatograms were run ascending on Whatman no. 1 Chromatography paper in a Shandon chromatography tank, although a few were run descending on Whatman no. 1 chromatography paper.

All the solvents were cleared from the chromatograms in the fume cupboard for 30 minutes except the formic acid - amyl alcohol - chloroform - water solvent, which was left to evaporate in the fume cupboard overnight. All the reagents were applied by dipping, except bromocresol green which was applied as a fine spray. Spots were allowed to develop at room temperature, except the sugars where the dipped chromatogram was heated at 95°C for a few minutes. The time of development at room temperature varied, from instantly (bromocresol green) to overnight (ninhydrin). Also with the ninhydrin dip, once the α - amino acids had developed the chromatogram was heated at 100°C for a few minutes to see if any further amino acids appeared.

The reagents (freshly prepared) used to identify specific organic compounds in the root preparations are shown below (Table 7).

Table 7.

The locating reagents.

Locating reagent	Ingredients	Quantity	Comments
Ninhydrin	Ninhydrin	0•2g	-
	Acetone	100ml	-
Isatin	Isatin	0•2g	-
	Acetone	100ml	-
Aniline - diphenylamine	1. Diphenylamine	4g	The ingredients added in the order 1 to 4.
	2. Acetone	200ml	
	3. Aniline	4ml	
	4. Phosphoric acid	20ml	
Bromocresol green	A { Bromocresol green	0•1g	-
	A { 99•5% ethanol	100ml	-
	A { N NaOH	Sufficient to turn the solution blue/green	-
	Mixture A	20ml	-
	Acetone	80ml	-

Continued .....

Table 7. (Cont.)

Locating reagent	Ingredients	Quantity	Comments
Sulphanilic acid	A { Sulphanilic acid	9g	-
	A { Concentrated HCl	90ml	-
	A { Distilled water	900ml	-
	B { Mixture A	25ml	Allow to stand for 4-5 mins.
	B { 5% sodium nitrite (in distilled water)	25ml	
	Mixture B	50ml	-
	Sodium carbonate, anhydrous, 10% in water.	50ml	-
Ehrlich	p-dimethylaminobenzaldehyde, 10% in concentrated HCl (w/v).	20ml	-
Ninhydrin - acetic acid	Ninhydrin, 0•2% in acetone	90ml	-
	Glacial acetic acid	10ml	-
Ferric chloride - ferricyanide	$FeCl_3$, 3% in water, diluted 10 times.	25ml	-
	$K_3Fe(CN)_6$, 3% in water, diluted 10 times.	25ml	-

The solvents and reagents used in the chromatography of the tomato root preparations are shown in Table 8.

Table 8.

Solvents and reagents used in the chromatography of

Chemical group	Quantity of excised tomato root preparation (expressed as mg dry weight of excised tomato root)			Solvent
	Exudate	Extract	Homogenate	
Amino acids	3	3	1•5	Butanol-acetic acid-water Phenol-ammonia-water
	3	3	3	Butanol-acetic acid-water
	3	3	3	Butanol-pyridine-water
	-	3	3	Butanol-acetic acid-water
	-	3	3	Butanol-acetic acid-water
Sugars	3	3	3	Butanol-acetic acid-water
	3	3	3	Butanol-pyridine-water
	3,1•5	6,3	-	Isopropanol-water
Organic acids	3	3	3	Butanol-acetic acid-water
	3	3	3	Butanol-pyridine-water
	3	3	3	Formic acid-amyl alcohol-chloroform-water
Ehrlich - positive substances.	6,3	6,3	6,3	Butanol-acetic acid-water
	6,3	6,3	6,3	Butanol-pyridine-water
	6	6	12,9,6,3	Butanol-acetic acid-water
	6	-	-	Butanol-pyridine-water Phenol-ammonia-water
	6,3	6,3	6,3	Isopropanol-ammonia-water
	-	6,3	6,3	Butanol-acetic acid-water
Phenolic acids	3	3	3	Butanol-acetic acid-water
	3	3	3	Isopropanol-ammonia-water
Phenols	6,3	6,3	6,3	Butanol-acetic acid-water

excised tomato root preparations.

Ratio of solvent	Ascending or descending, time and distance.	Locating reagent
60:12:25	10 hours Ascending	Ninhydrin
160:1:40	Overnight 2way	
60:12:25	Ascending, 8 hours, 16cm	Ninhydrin
60:60:60	Ascending, 8 hours, 16cm	Ninhydrin
60:12:25	Descending, 22 hours, 41cm	Isatin
60:12:25	Descending, 24 hours, 41cm	Ninhydrin
60:12:25	Ascending, 8 hours, 16cm	Aniline-diphenylamine
60:60:60	Ascending, 8 hours, 16cm	Aniline-diphenylamine
160:40	Descending, 48 hours, -	Aniline-diphenylamine
60:12:25	Ascending, 8 hours, 16cm	Bromocresol green
60:60:60	Ascending, 8 hours, 16cm	Bromocresol green
30:80:80:80	Descending, 16 hours, 41cm	Bromocresol green
60:12:25	Ascending, 8 hours, 16cm	Ehrlich
60:60:60	Ascending, 8 hours, 16cm	Ehrlich
60:12:25	Ascending, 10 hours, 18cm	Ehrlich and long wave UV
60:60:60	10 hours Ascending	Ehrlich
160:1:40	Overnight 2-way	
200:10:20	Ascending, 10 hours, 18cm	Ehrlich
60:12:25	Descending, 22 hours, 41cm	Ehrlich
60:12:25	Ascending, 8 hours, 16cm	Sulphanilic acid
200:10:20	Ascending, 10 hours, 18cm	Sulphanilic acid
60:12:25	Ascending, 8 hours, 16cm	Ferric chloride - Ferricyanide reagent

Continued .....

Table 8. (cont.)

Chemical group	Quantity of excised tomato root preparation (expressed as mg dry weight of excised tomato root)			Solvent
	Exudate	Extract	Homogenate	
Tryptamines	–	3	3	Butanol-acetic acid-water
Vitamins	3	3	3	Butanol-acetic acid-water
	3	3	3	Butanol-pyridine-water
	0•1mg pine root exudate (collected for 3 weeks)			Butanol-acetic acid-water
	0•1mg pine root exudate (collected for 3 weeks)			Butanol-pyridine-water

Ratio of solvent	Ascending or descending, time and distance.	Locating reagent
60:12:25	Ascending, 10 hours, 10cm	Ninhydrin-acetic acid
60:12:25	Ascending, 8 hours, 16cm	Long wave UV
60:60:60	Ascending, 8 hours, 16cm	Long wave UV
60:12:25	Ascending, 8 hours, 16cm	Long wave UV
60:60:60	Ascending, 8 hours, 16cm	Long wave UV

VITAMIN ASSAY.

Since only a few vitamins can be identified using long wave ultra-violet light, vitamin requiring microorganisms were used, to determine the presence of any vitamin in exudate, extract and homogenate of excised tomato roots. This technique is likely to be more sensitive than that using long wave ultra-violet light as a locating reagent in chromatography.

1. Assays with *Escherichia coli* mutants.

In order to determine the presence of niacin and thiamin, two vitamin requiring mutants, *Escherichia coli* nia⁻ (8110, obtained from the Tory Research Station, Aberdeen) and *Escherichia coli* thi⁻ (respectively) were used.

An overnight culture of each bacterium was prepared by inoculating 4ml of nutrient broth, sterilized in centrifuge tubes, with bacteria cells.

Nutrient broth.

Nutrient broth no. 2 (Oxoid)	25g
Distilled water	to 1000ml

pH 7·5

Autoclaved at 15 p.s.i. for 15 minutes.

The tubes were incubated at 37°C overnight, then centrifuged at 5,000 r.p.m. for 20 minutes and the supernatant discarded. The cells were then re-suspended in 4ml of sterile buffer, re-centrifuged and re-suspended.

Buffer.

$Na_2HPO_4 \cdot 2H_2O$	9•0g
KH_2PO_4	3•0g
NaCl	4•0g
$MgSO_4 \cdot 7H_2O$	0•2g
Distilled water	to 1000ml

The buffer was autoclaved at 15 p.s.i. for 15 minutes and Analar reagents were used where available.

10^{-2} and 10^{-4} dilutions of each bacterium were made, using the buffer as the dilutant.

0•5ml of excised tomato root extract (equivalent of 6•2mg dry weight of root), sterilized by micropore filtration was aseptically transferred to 3cm sterile petri dishes to which 5ml of cooled molten minimal agar was added and the two were mixed together.

Minimal agar.*

Glucose	2.0g
NH_4Cl	5·0g
NH_4NO_3	1·0g
$NaSO_2$ anhydrous	2·0g
K_2HPO_4 anhydrous	3·0g
$MgSO_4 \cdot 7H_2O$	0·1g
Purified agar (Oxoid)	15g
Distilled water	to 1000ml

pH 7·2

Analar reagents were used where available and the medium was autoclaved at 15 p.s.i. for 15 minutes.

This was repeated six times (three treatments for E. coli thi⁻ and three for E. coli nia⁻) and then repeated for homogenate and exudate (from live roots collected in distilled water) of excised tomato roots. Also six plates of minimal agar containing 1ug/ml of niacin, and three containing 1ug/ml of thiamin were prepared, and also twelve plates with no vitamins or sample, to use as controls.

Two drops of a 10^{-2} dilution of E. coli thi⁻ were spread on the surface of the minimal agar containing extract, homogenate or thiamin and on control agar.Two drops of a 10^{-4} dilution were spread over the surface of agar containing exudate or thiamin or control agar This same procedure was repeated with E. coli nia⁻ and all the plates

* Each salt was dissolved in cold water in the above order, waiting until the previous salt dissolved before adding the next. As a light precipitate forms, the solution was filtered, before mixing with the agar and glucose solution.

were incubated at 37°C overnight.

Samples of niacin and thiamin were also run on chromatograms in BuA and BuP in order to determine their Rf fraction, using this assay. 10ug loadings were used and after running, the chromatograms were divided into Rf fractions (1 - 10). Each chromatogram fraction was sterilized under the ultra - violet light and then aseptically transferred to the minimal agar in petri dishes spread with two drops of 10^{-4} dilution of cells (E. coli thi⁻) or two drops of a 10^{-2} dilution (E. coli nia⁻). Three plates of niacin and three of thiamin and six without vitamins were used as controls. Again the petri dishes were incubated at 37°C overnight.

2. Assays with Aspergillus nidulans mutants.

The presence of biotin and pyridoxine in the excised tomato root preparations was determined using the vitamin requiring mutants Aspergillus nidulans bio⁻ and A. nidulans pyr⁻, both fungi also requiring adenine.

Cultures of these fungi were maintained on V8 medium

V8 medium.

V8 juice	200ml
$CaCO_3$ (Analar)	3g
Agar agar (Oxoid)	15g
Distilled water	to 1000ml

0·5ml of excised tomato root exudate (from live roots collected in distilled water), extract or homogenate (i.e. equivalent to 6·2mg dry weight of root), all sterilized by

micropore filtration, also pyridoxine and biotin (sterilized by autoclaving at 5 p.s.i. for 10 minutes) were incorporated into 3cm agar plates containing 5ml sterile BIA (see page 52 for details) with adenine (100ug/ml). Control plates contained no root preparation or vitamins.

Agar plugs (5mm diameter) from the edge of the mycelium of A. nidulans nis⁻, or A. nidulans pyr⁻ were transferred aseptically to the petri dishes.

Biotin was run on a chromatogram in order to determine its Rf fraction in BuA and BuP, using the same procedure as niacin and thiamin (see above, page 118). Inoculation was by means of agar plugs cut from the perifery of the mycelium and then transferred to petri dishes containing BIA with adenine.

The Rf fraction of pyridoxine run in BuA and BuP was determined by long wave ultra - violet light fluorescence.

IDENTIFICATION OF RIBOFLAVIN USING THE MASS SPECTROMETER.

In order to determine whether riboflavin could be the substance(s) which stimulates the root isolate M13, and which is present in pine seedling root and excised tomato root exudate, extract and homogenate, samples of extract were prepared for analysis in the Mass Spectrometer. The excised tomato root extract was prepared using the same method as the chromatographic bioassay (see page 99). 1•5ml of extract (equivalent of three excised tomato roots) was streaked onto the origin and run in BuA or BuP. The chromatogram was cut into the usual Rf fractions (1 to 10) and then the M13 growth stimulatory fractions were eluted either individually (fractions 1 and 7 after fractionation in BuP and fraction 8 after fractionation in BuA) or combined (fractions 0 – 4 after fractionation in BuA) in distilled water overnight at 6°C. Each elutant was then evaporated to dryness and finally taken up in two drops (0•04ml) of ethanol.

This technique was repeated using riboflavin (1ug) applied as a spot instead of excised tomato root extract. The riboflavin spots were identified on the chromatogram under the long wave ultra - violet light and were then eluted and concentrated as before.

These samples as well as a very dilute solution of riboflavin, consisting of one crystal of riboflavin suspended in two drops of ethanol, were then analysed by the Mass Spectrometer (Micromass 12, V.G. Micromass Ltd.) and the results were compared.

GROWTH EXPERIMENTS WITH COMPOUNDS IDENTIFIED IN EXCISED TOMATO ROOT PREPARATIONS.

1. Amino acids.

The amino acids tentatively identified in excised tomato root exudate (from live roots, collected in distilled water), extract and homogenate were tested both individually and in combination, on the growth of M13 in BLM plus vitamins, and MLM. The quantities of amino acids used were either those concentrations used in the MLM, or if the amino acid was not present in this medium, then a volume equal to that of an amino acid (present in MLM) of a similar spot size was used. (See Table 9 below). Each flask also contained 0·5ml of filtered fungal macerate, of M13, (prepared as on pages 70 and 65(b)) and they were incubated at 25°C for 14 days.

Table 9.

Concentrations of amino acids used in growth experiments.

Amino acid	Concentration (ug/ml of nutrient medium)
Isoleucine	24•5
Methionine	17•0
Valine	39•5
Alanine	9•5
*Glycine	2•5
Lysine	30•0
Histidine	12•5
*Tyrosine	165•0
Glutamine	150•0
*Hydroxyproline	5•0
Asparagine	20•0
Serine	2•5
Aspartic acid	20•5
Cysteine	20•0

* These amino acids although included in this experiment were not positively identified in any of the excised tomato root preparations.

2. Sugars.

The three sugars, glucose, fructose and sucrose, which were present in excised tomato root extract and homogenate were tested both individually and as a mixture on the growth of M13 in MLM. They were added to flasks containing 50ml MLM, after steam sterilization to give a final concentration of sugars in the medium of 2•05% with the individual sugars or 2•15% with the sugar mixture. Each flask also contained 0•5ml of filtered fungal macerate, prepared as on pages 70 and 65(b) and they were then incubated at 25°C for 14 days.

3. Riboflavin.

The vitamin riboflavin, which appeared to have the same Rf fractions as the M13 growth stimulatory factor(s), and which was identified in all excised tomato root preparations and pine seedling root exudate (collected for 3 weeks) was tested on the growth of M13 in MLM. Either 10ug, 30ug or 100ug of micropore filtered riboflavin was added to 50ml of MLM containing 0•5ml of filtered fungal macerate (see pages 70 and 65(b)) in 250ml Erlenmeyer flasks. They were then incubated at 25°C for 14 days.

RESULTS

ISOLATION OF THE FUNGI FROM ECTOMYCORRHIZAL ROOTS OF PINUS SYLVESTRIS AND SPOROPHORES.

Suspected ectomycorrhizal fungi emerged from sterilized ectomycorrhizal roots approximately two weeks after plating them out onto nutrient agar (e.g. M19 and M25). However some did not grow until after a time lapse of four weeks e.g. M13, M31 and M51, eight weeks e.g. M5 and M6 or even sixteen weeks e.g. M88, M89, M90 and M91. Often two morphologically distinct fungi appeared to emerge from one ectomycorrhiza, this also being observed by Zak and Bryan (1963).

All these fungi grew very slowly in pure culture, their growth rate relating to the time of emergence. Those emerging 2 weeks after sterilization grew much faster than those emerging 16 weeks after sterilization. This was true for all the isolates except those having a black mycelium, these growing much faster than the rest of the fungal isolates. All isolates were septate but no clamp connections could be detected in any of them.

Three of the isolates were black in colour on BIA, the rest being either white, grey or varying shades of brown, and most of them showed some degree of variation in colour between the old and the new parts of the mycelium when grown on agar.

A number of the fungi isolated from the ectomycorrhizal roots appeared to be morphologically similar on BIA as shown in Table 10 below:-

Table 10.

Groups of root isolates which appear morphologically similar on BIA.

Group	Root isolates which appear morphologically similar on BIA
1	M40B, M41, M44A, M44B, M49, M53, M59A
2	M59B, M60, M65
3	M79, M80
4	M51, M56
5	M52, M57
6	M72, M73

A selection of these root isolates were sent to the Commonwealth Mycological Institute at Kew, for identification, where it was found that most of them remained sterile despite near-UV treatment for several weeks. Only two isolates, M78 and M12 were identified as Verticillium psalliotae Treshow and an Acremonium sp. respectively.

There was little variation between the percentage number of fungi isolated from ectomycorrhizae collected from Brandon Forest, on any medium used, after sterilization with sodium hypochlorite, all giving a yield of approximately 50% recovery from the ectomycorrhizal roots. Those collected from Angley Woods all gave a yield of approximately 20% (see Table 11).

Yields obtained, following sterilization of roots with 100 vols hydrogen peroxide were generally very low (less than 4%), most being obtained on MEA plates. Using this method of sterilization slightly more isolates were obtained from Angley Woods ectomycorrhizal roots than those from Brandon Forest (see Table 11).

Table 11.

Percentage number of suspected ectomycorrhizal fungi from sterile ectomycorrhizal roots.

Steri-lant	Source of ecto-mycorrhizae	Medium	Origional number of roots	Final number of roots	Number of suspected ecto-mycorrhizal fungi	Percentage number of suspected ecto-mycorrhizal fungi
H_2O_2	Thetford	MEA	512	384	7	1•8
H_2O_2	Thetford	BIA	480	448	1	0•3
H_2O_2	Thetford	TM	416	256	1	0•3
H_2O_2	Thetford	MMN	320	288	0	0•0
H_2O_2	Angley	MEA	928	288	11	3•8
H_2O_2	Angley	BIA	752	496	2	0•4
H_2O_2	Angley	TM	272	224	1	0•5
H_2O_2	Angley	MMN	192	128	0	0•0
Na hypo	Thetford	H+strep	360	265	138	52•1
Na hypo	Thetford	H+Y+strep	360	283	133	47•0
Na hypo	Thetford	MEA	360	240	116	48•3
Na hypo	Angley	H+strep	448	81	19	23•5
Na hypo	Angley	H+Y+strep	448	63	13	20•6
Na hypo	Angley	MEA	432	36	6	16•7

Key

MEA=Malt extract agar

TM=Taylor's medium

BIA=Basic ion agar

MMN=Modified Melin-Norkrans medium

Strep=Streptomycin

H_2O_2=100vols hydrogen peroxide

H=Hagem medium

Na hypo=Sodium hypochlorite (1%)

H+Y=Hagem medium+yeast extract

Isolates of _Boletus variegatus_ 1 and 2, _Boletus badius_, _Boletus bovinus_, _Boletus luteus_ and _Amanita rubescens_, were obtained from sterile pieces of sporophore tissue. These were maintained on BIA and like the root isolates they all exhibited slow growth rates.

Since the yield of isolates from ectomycorrhizal roots obtained with 100vols hydrogen peroxide was much lower than with sodium hypochlorite, the effect of a diluted solution of hydrogen peroxide was observed on the growth of M13 and also _Boletus variegatus_ 1.

From Fig. 6. it appears that hydrogen peroxide has a strong inhibitory effect on M13 and relatively little effect on _B.variegatus_ 1 especially at the lower concentrations. The implication is that M13 had recovered to a "high" growth rate following its initial isolation from ectomycorrhizal roots using hydrogen peroxide as a sterilant.

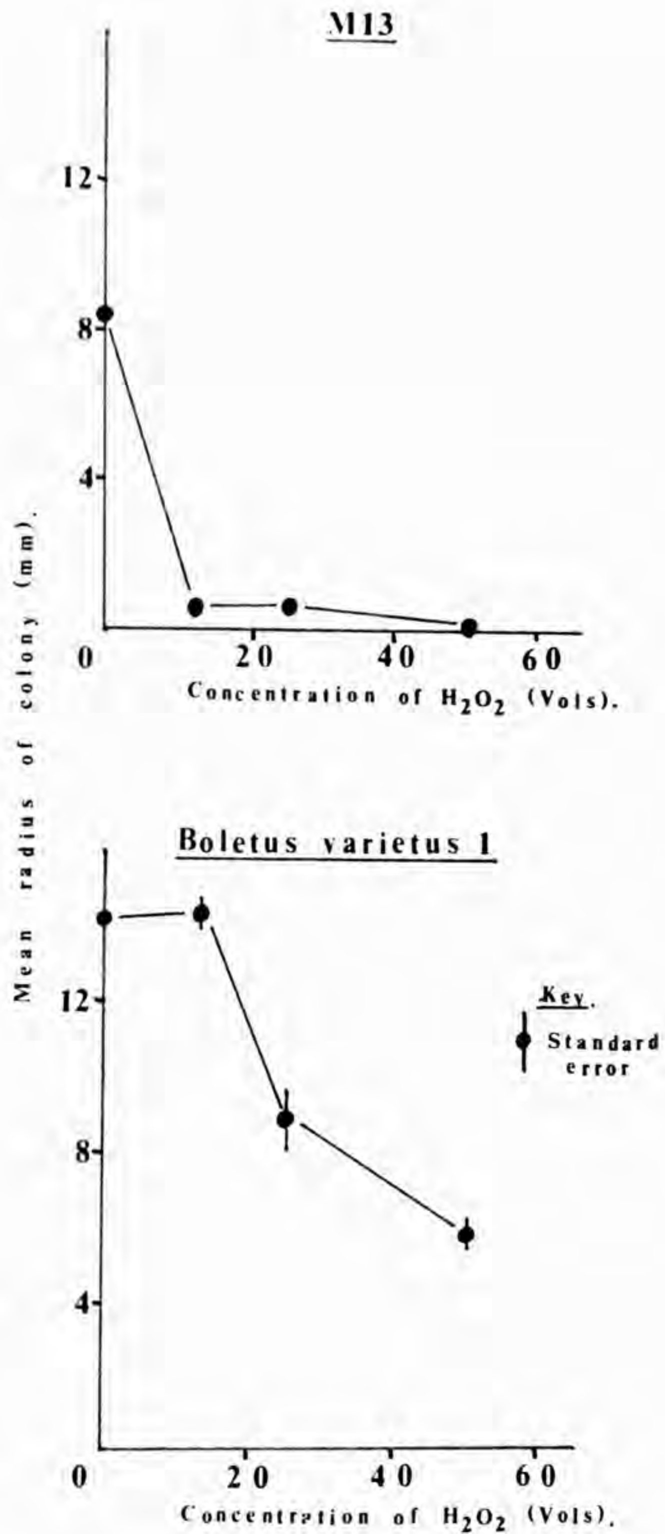


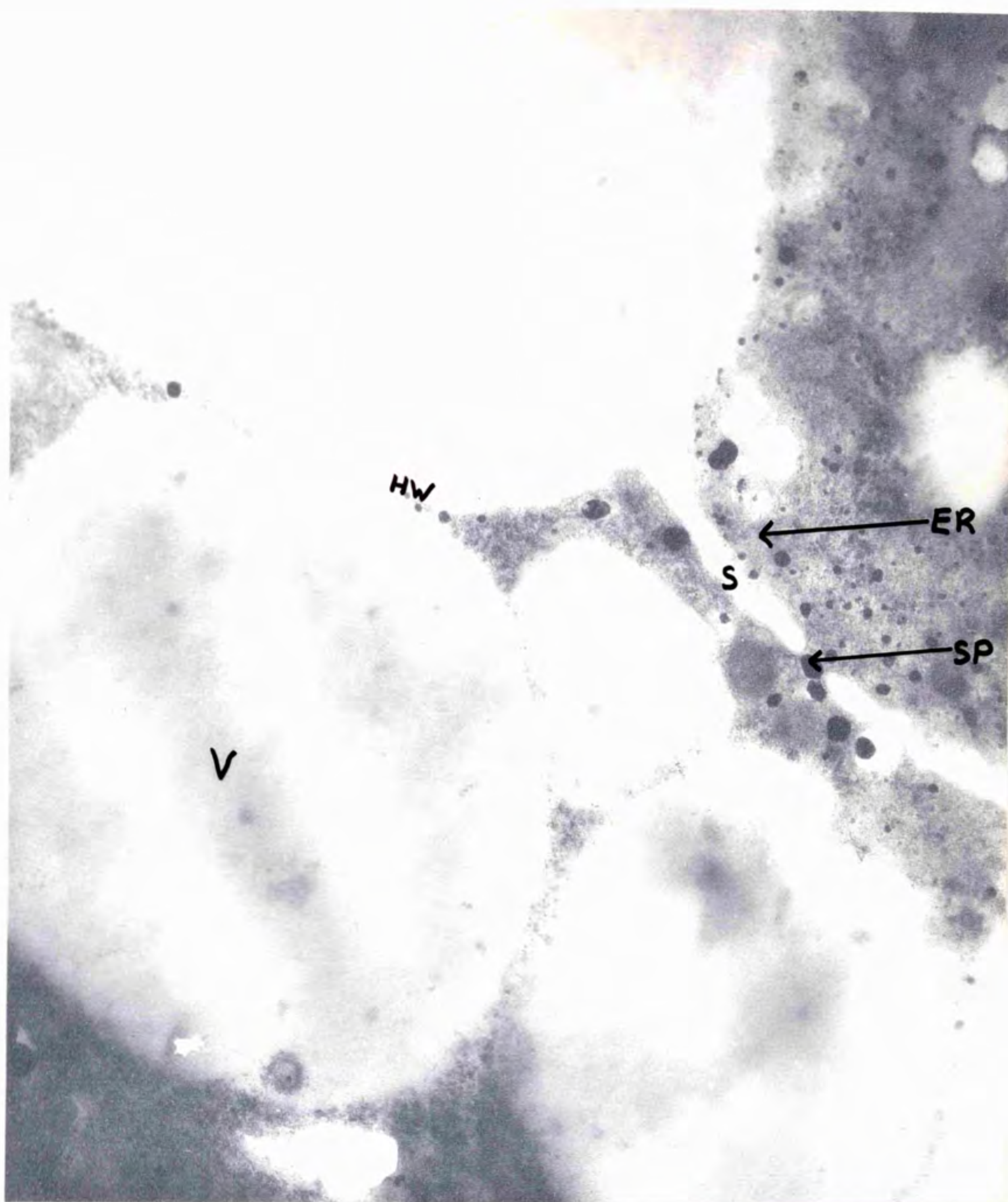
Fig. 6. The effect of hydrogen peroxide on the growth of Boletus variegatus 1 and M13.

ULTRASTRUCTURE OF M1, M8, M23 AND BOLETUS LUTEUS.

On examination of the three root isolates under the electron microscope (see Figs. 7, 8, 9) the hyphae of M1, M8 and M23 appeared more vacuolated than those found in Boletus luteus, the hyphae often being nearly filled with vacuoles. In some of these vacuoles, deposits of an unknown substance occurred which often filled most of the vacuole. Deposited throughout the hyphae of these isolates were a large number of electron dense, unidentifiable bodies, which could be either oil bodies, glycogen granules or the vesicles mentioned by Foster and Marks (1966). Abundant endoplasmic reticulum and mitochondria were also present. The septa seen in these hyphae were complete, and generally no pores or evidence of dolipore septa were visible, either because the sections were not taken across the centre of the septa, or because there were none present. In one isolate, M1, there was a simple pore across the centre of the septum, the cytoplasm from one cell connecting with that of the next cell, although the actual pore was blocked by an electron dense body (Fig. 7).

In B. luteus complete septa were seemingly quite abundant (Fig. 10) but a few dolipore septa were observed (Fig. 11). The parenthosome, covering the dolipore septa and appearing to be continuous with the endoplasmic reticulum (Moore, 1965), was perforated. Also a plug of electron dense material blocked both openings of the dolipore, an observation recorded also for Polystictus (Gibardt, 1961). Abundant endoplasmic reticulum, mitochondria and ribosomes were also present in the hyphae and in the vacuoles there were often light coloured spherical inclusions (Fig. 10).

The electron micrographs seem to indicate that M1 could belong



KEY

Magnification x 80,000

V=Vacuole.

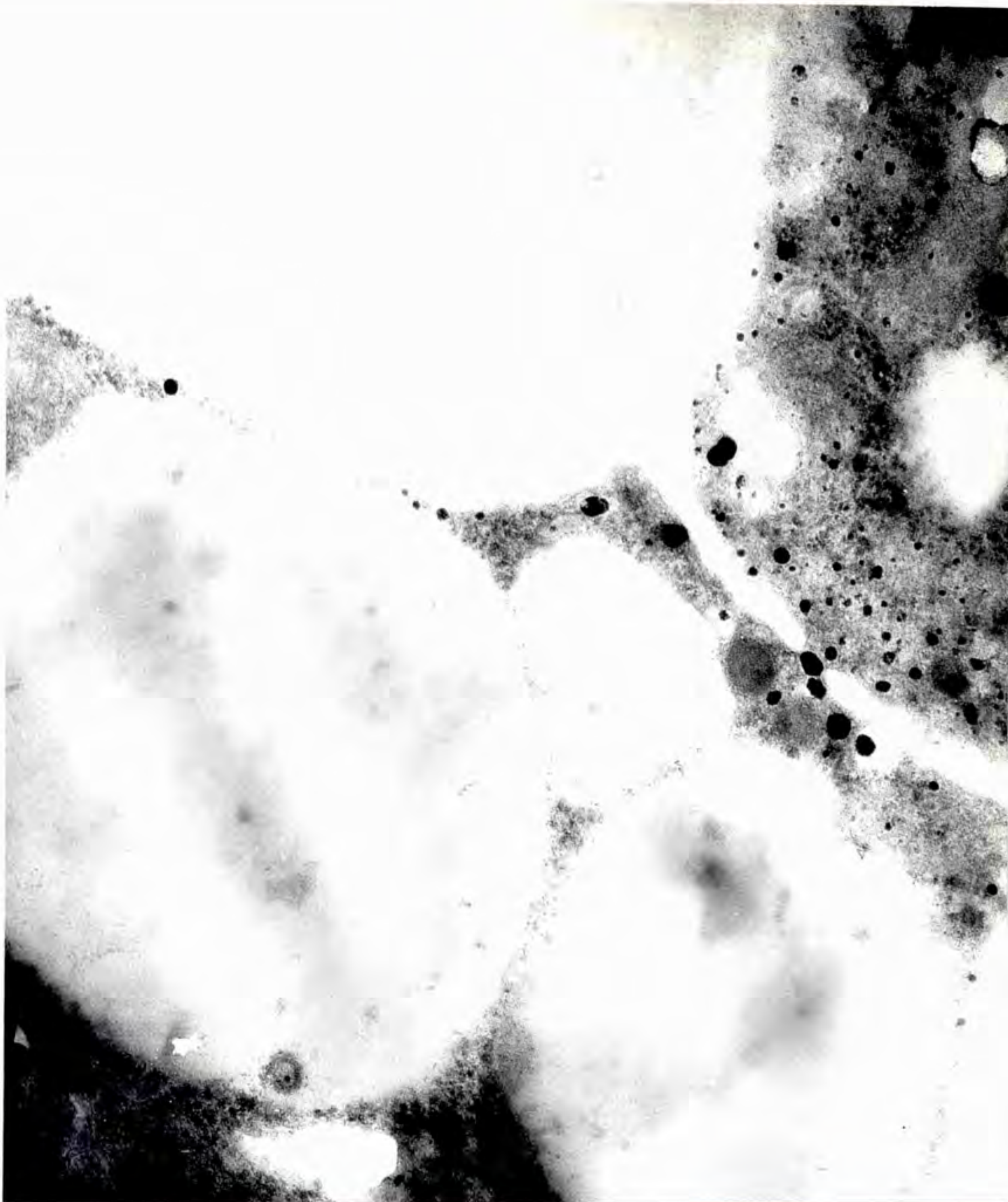
ER=Endoplasmic reticulum.

S=Septum.

SP=Simple pore blocked by an electron dense body.

HW=Hyphal wall.

Fig. 7. Section through a hypha of M1.



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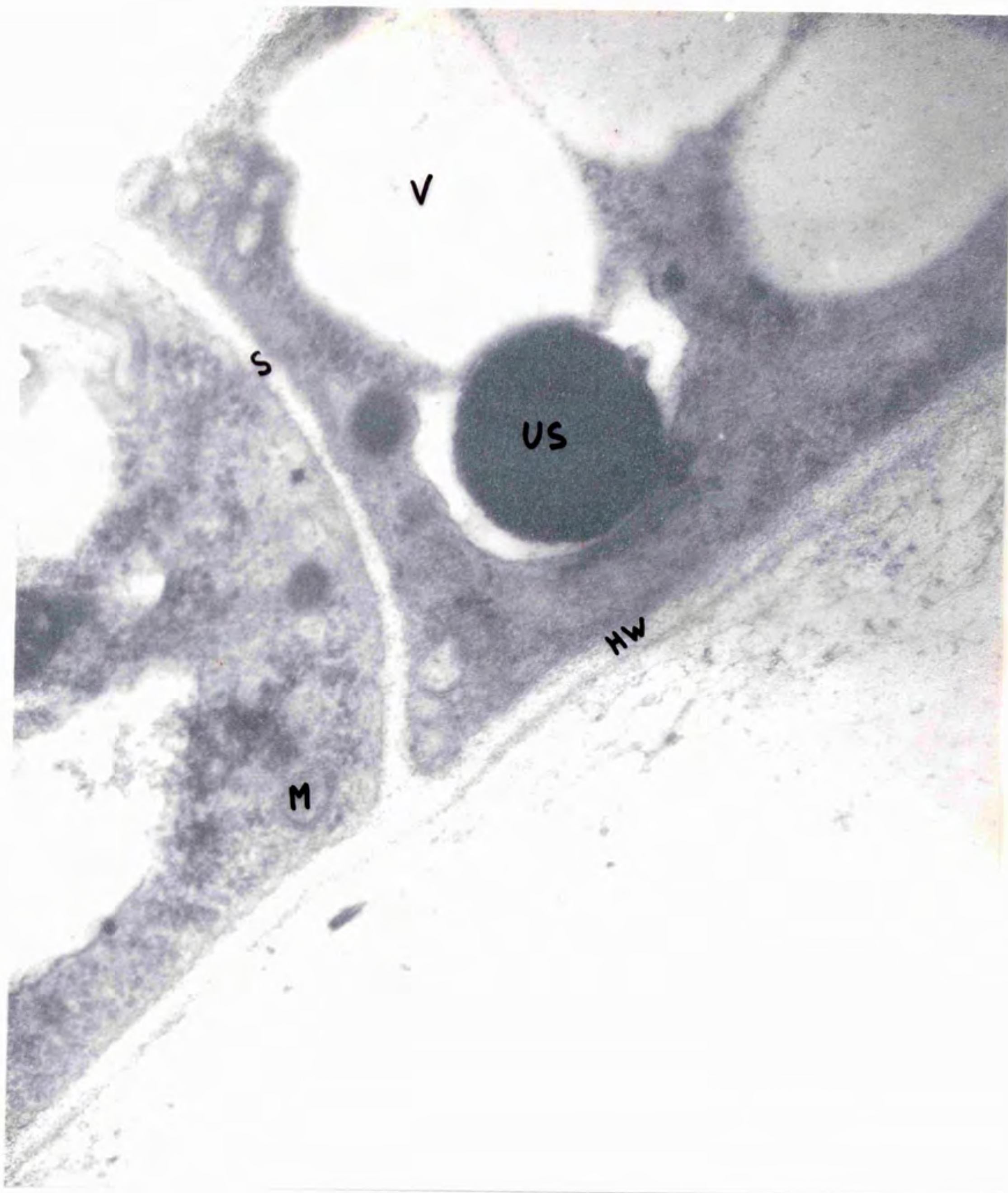


KEY

Magnification x 80,000

V=Vacuole.

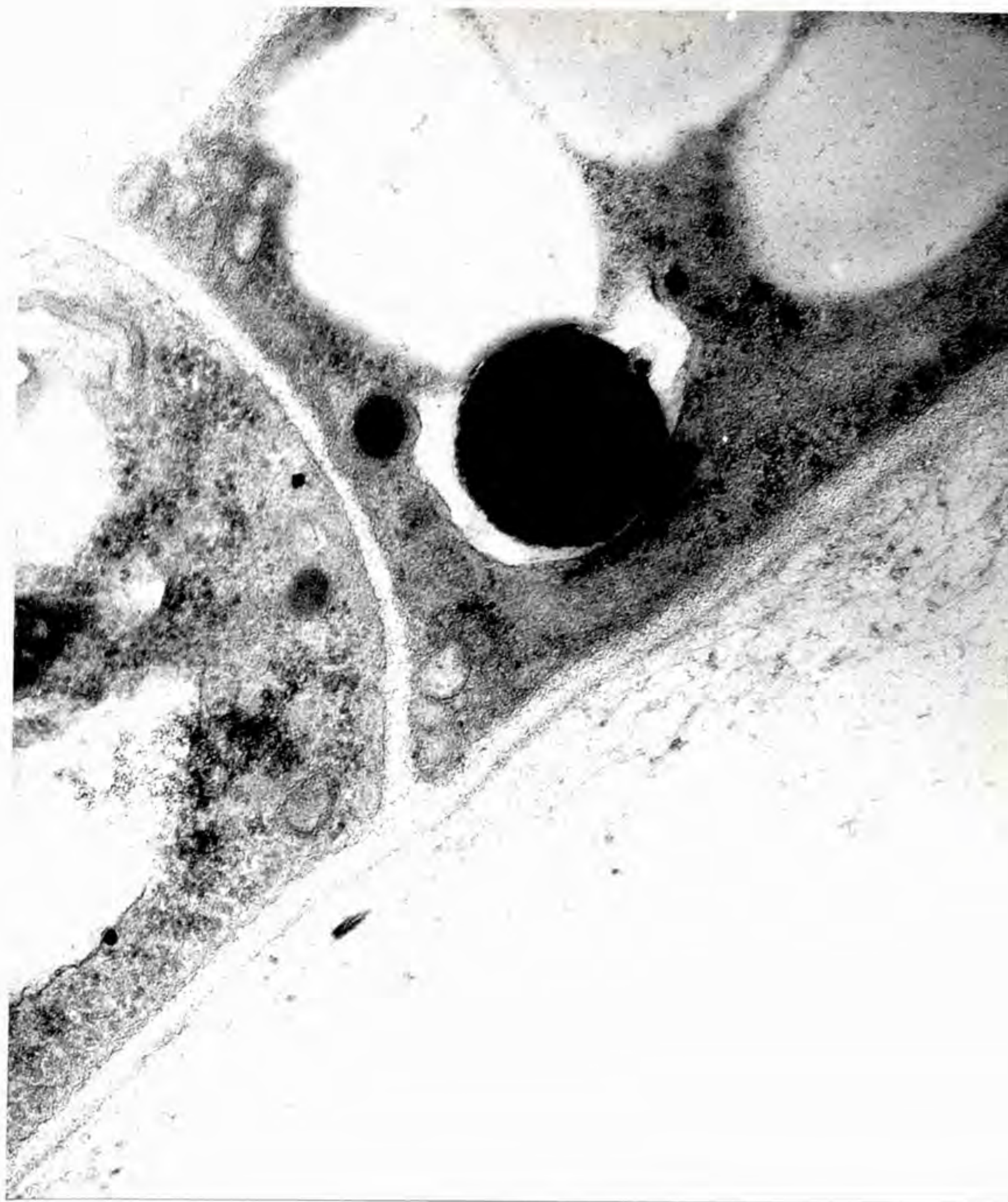
S=Septum.

M=Mitochondrion.

HW=Hyphal wall.

US=Unknown substance deposited in a vacuole.

Fig. 8. Section through a hypha of M8.



KEY

V=Vacuole.

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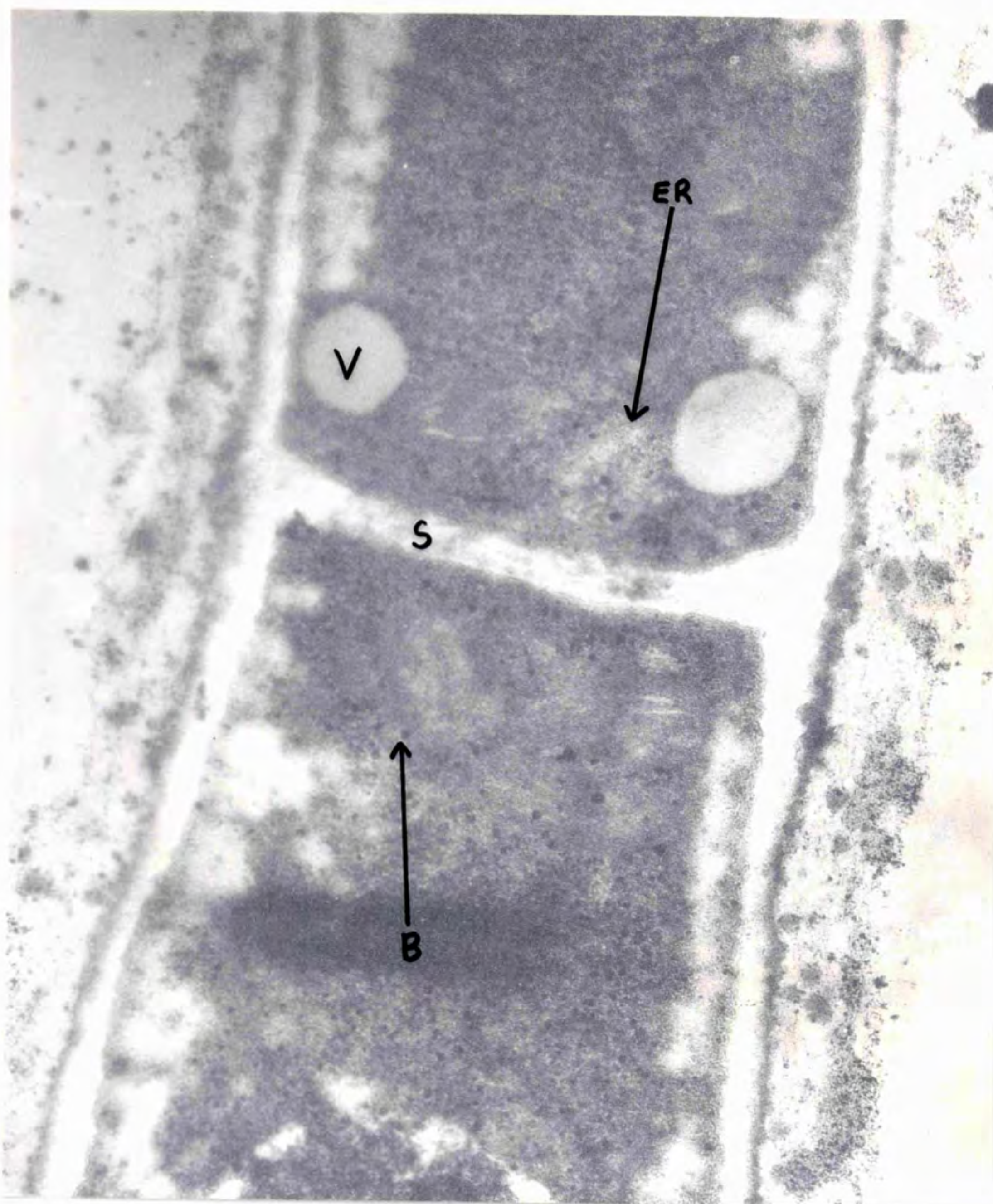
Magnification x 80,000

Fig. 8. Section through a hypha of M8.



KEY

Magnification x 80,000

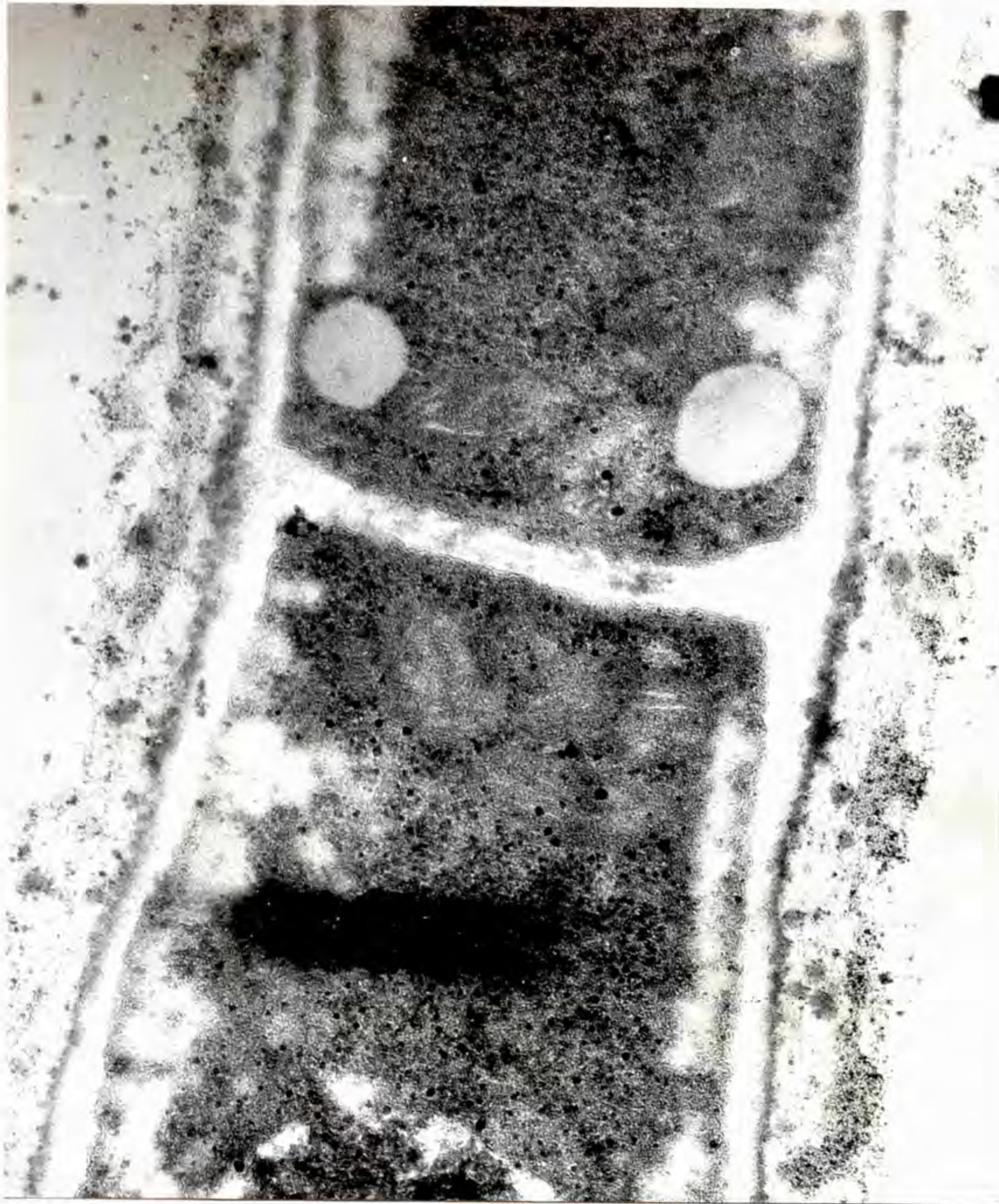
ER=Endoplasmic reticulum.

S=Septum.

V=Vacuole.

B=Electron dense body.

Fig. 9. Section through a hypha of M23.



KEY

ER=Endoplasmic reticulum.

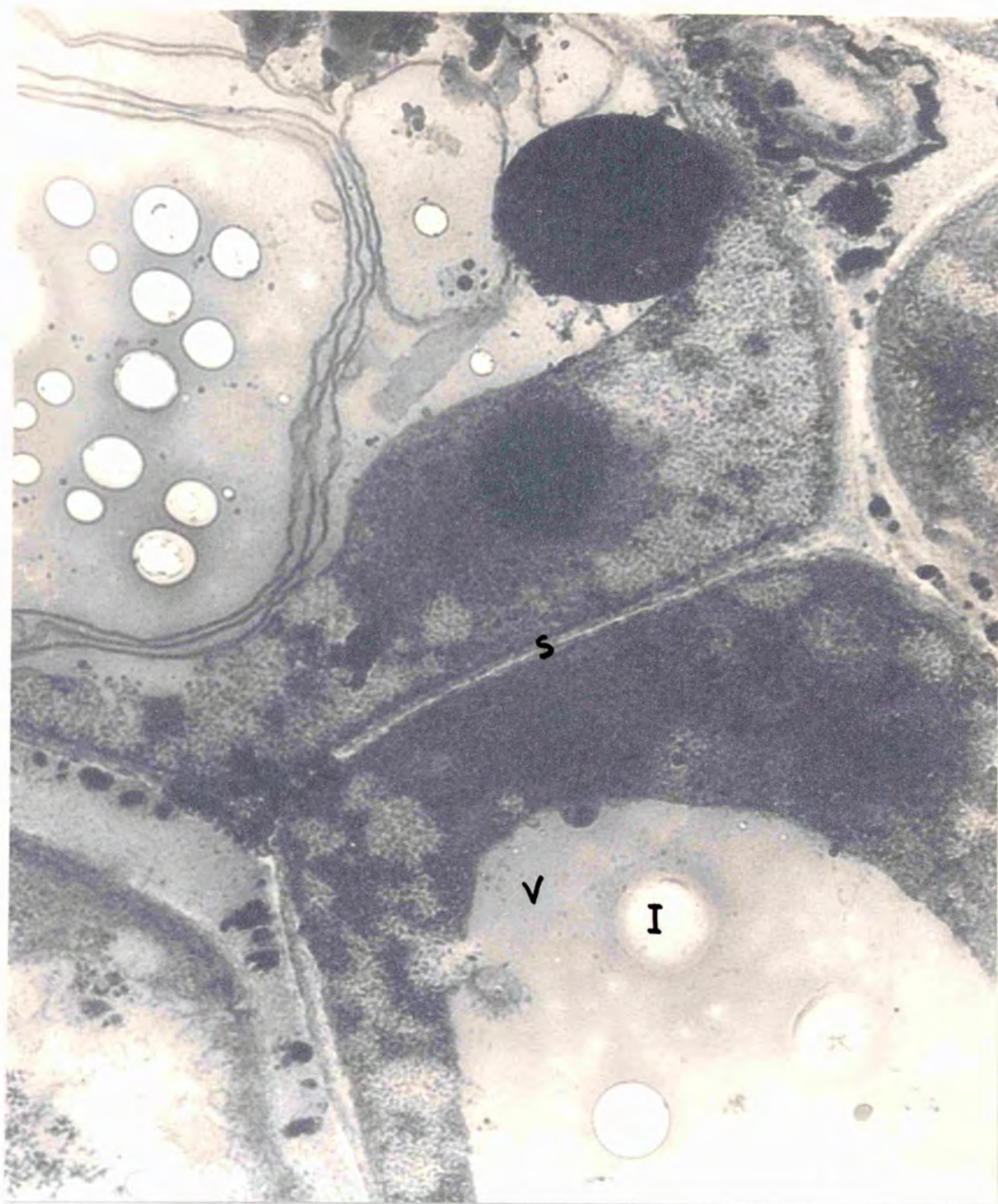
S=Septum.

V=Vacuole.

B=Electron dense body.

Magnification x 80,000

Fig. 9. Section through a hypha of M23.



KEY

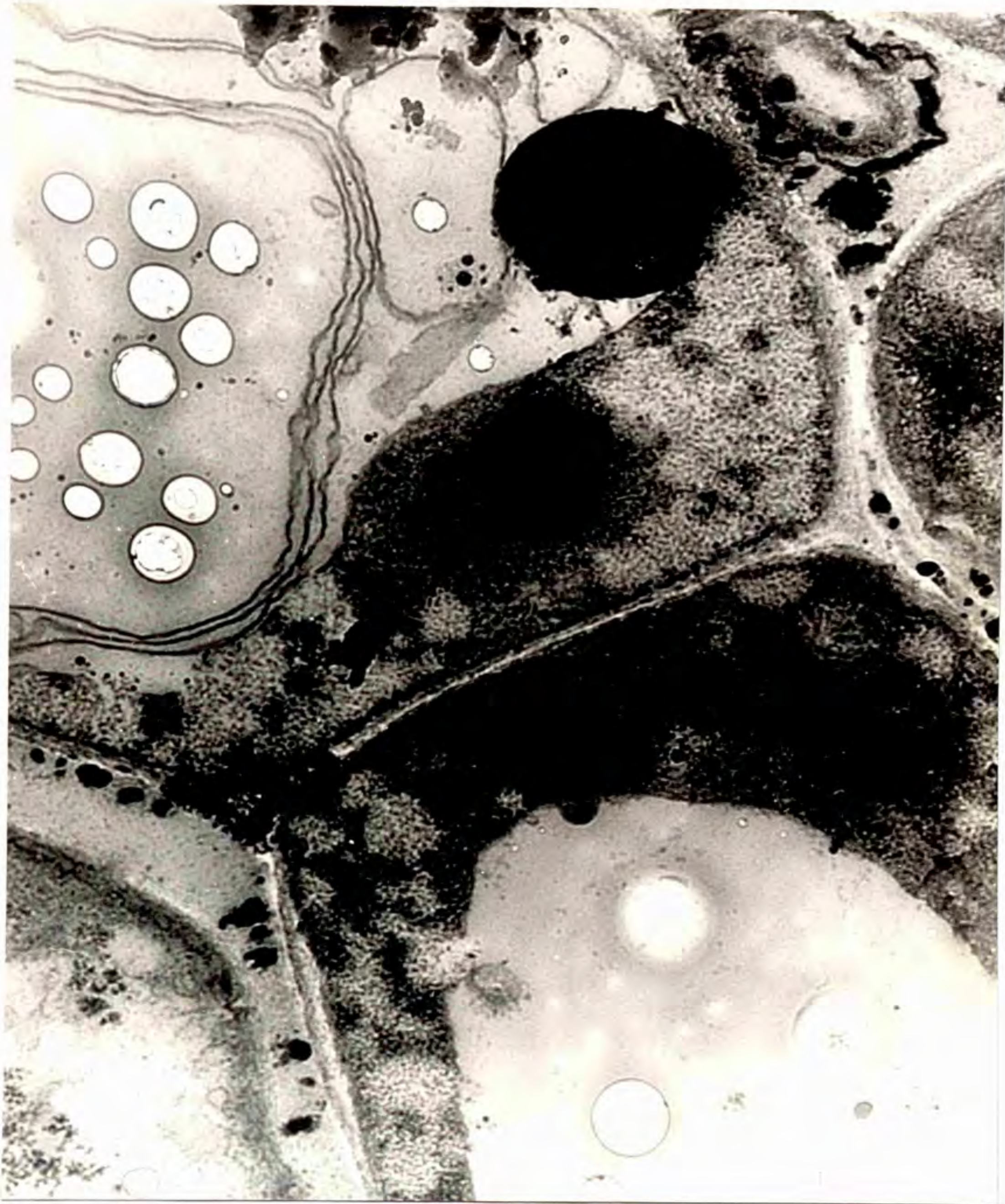
S=Septum.

V=Vacuole.

I=Spherical inclusion.

Magnification x 32,000

Fig. 10. Section through a hypha of Boletus luteus.



KEY

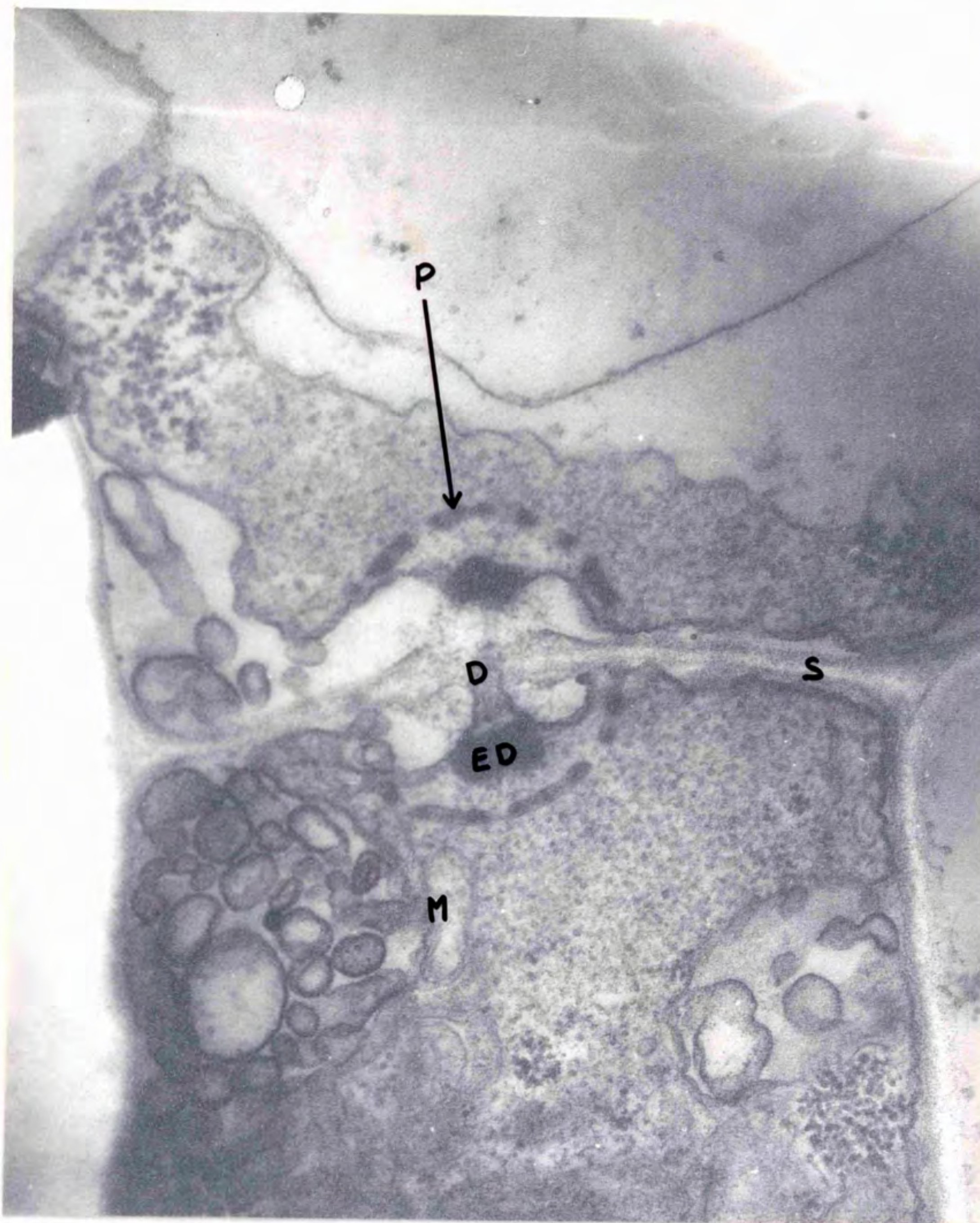
S=Septum.

V=Vacuole.

I=Spherical inclusion.

Magnification x 32,000

Fig. 10. Section through a hypha of Boletus luteus.



KEY

Magnification x 80,000

P=Parenthosome.

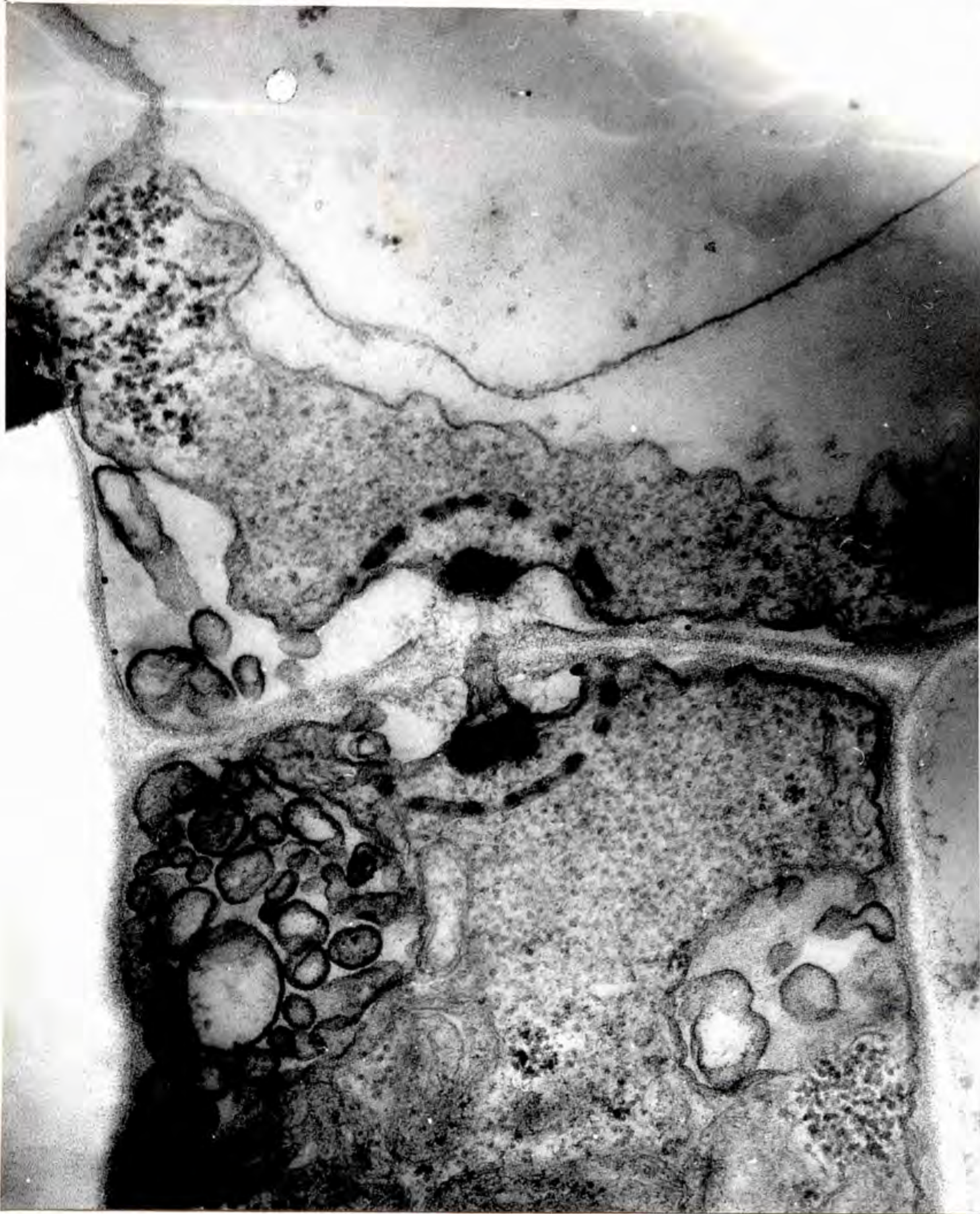
D=Dolipore.

S=Septum.

ED=Electron dense material blocking dolipore.

M=Mitochondrion.

Fig. 11. Section through a hypha of Boletus luteus. Transverse section through a dolipore septum.



KEY

Magnification x 80,000

P=Parenthosome.
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S=Septum.
ED=Electron dense material blocking dolipore.
M=Mitochondrion.

Fig. 11. Section through a hypha of Boletus luteus. Transverse section through a dolipore septum.

therefore, to the Ascomycetes, since a simple pore was observed in one septum, but no evidence of either a simple pore or dolipore could be found in M8 or M23 and hence there is no conclusive evidence for placing either of these fungi in the Basidiomycetes or Ascomycetes.

SYNTHESIS OF ECTOMYCORRHIZAE.

Melin's (1921) method for the synthesis of ectomycorrhizae with modifications designed to maintain the flasks in a controlled environment was the only method which was successful in the synthesis of ectomycorrhizae *in vitro*, and then only *Boletus luteus* formed ectomycorrhizae.

Fortin's method (1966) using various substrates and also different dilutions of the mineral medium was totally unsuccessful, although lateral root growth did occur in a number of the explants. Growth of the fungus in the substrate was always abundant especially in the region of the root and the fungus grew on the surface of the root. When lanolin was applied to the open end of the vials, no growth of the fungus occurred in the organic medium. Addition of further inoculum or using more inoculum or extending the incubation period did not result in ectomycorrhizal formation.

In some cases (M3 and *Boletus luteus* in perlite) secondary lateral roots were branched dichotomously, but there was no evidence of ectomycorrhizal formation, since they were not swollen and no fungal mantle or Hartig net had formed (Fig. 12).

Many root isolates had no effect on the number of laterals formed nor their length, when grown in a substrate other than perlite.

The results (see Appendix B) suggest that substrate and media affected the formation of the lateral roots. Water as a "medium" added to the humus produced very little lateral root formation, and often in dilute mineral medium fewer lateral roots formed than in the normal concentration of mineral medium. The perlite gave the best results for growth of lateral roots. Sand as a substrate tended to dry out very quickly whereas perlite held its moisture for longer periods.

Magnification x 2·5

Fig. 12. Fortin's method for the synthesis of ectomycorrhizae in vitro.
Formation of dichotomous lateral roots on an explant of Pinus sylvestris after inoculation with Boletus luteus.

The method of ectomycorrhizal synthesis which was used by Pachlewski (Pachlewski, 1967; Pachlewska, 1967; Pachlewski and Pachlewska, 1967) was also unsuccessful, although there was a slight increase in lateral root formation when the tubes had been inoculated with the fungal isolates.

Some inoculated seedlings (with mycelial agar plugs and later a fungal macerate), after six months produced dicotomous secondary lateral roots with M1, M3, M7, Boletus luteus and Boletus variegatus 1, but again there was no evidence of penetration of the fungus into the root to form ectomycorrhizae (see Fig. 13). In the tubes where mycelial agar plugs had been used as a source of inoculum the fungus grew only on the surface of the agar, but when a further inoculum of fungal macerate was added, the hyphae grew profusely throughout the agar and also around the roots of the pine seedlings. Still no ectomycorrhizae formed.

The method used by Melin and modified by Marx and Zak (1965) using perlite and a mixture of peat and perlite as substrates was also unsuccessful.

(Tube viewed from below) Magnification x 2·5

Fig. 13. Pachlewski's method for the synthesis of ectomycorrhizae in vitro.
Formation of dichotomous secondary lateral roots six months after the inoculation of seedlings of Pinus sylvestris with Boletus luteus.

Table 12.

Growth of Pinus sylvestris seedlings enclosed in 2-litre flasks in two different substrates with various root isolates.

Fungus	Substrate	Period of incubation (months)	Root mean dry weight between replicate flasks (2) mg	Standard error	Shoot mean dry weight between replicate flasks (2) mg	Standard error
M1	Perlite	25	159•7	(36•8)	333•3	(94•6)
M1	Perlite	3	80•7	← not available →		
M3	Perlite	25	164•0	(128•6)	330•0	(191•2)
M3	Perlite	3	37•5	← not available →		
M7	Perlite	25	139•2	(25•2)	275•8	(68•2)
M7	Perlite	3	22•4	← not available →		
M8	Perlite	25	124•1	(24•3)	277•1	(60•1)
M8	Perlite	3	22•5	← not available →		
*Control	Perlite	25	53•9	-	424•1	-
M1	Pt+perlite	25	64•7	(3•1)	347•2	(28•8)
M1	Pt+perlite	3	15•3	← not available →		
M3	Pt+perlite	25	177•9	(74•2)	707•3	(136•5)
M3	Pt+perlite	3	16•7	← not available →		
M7	Pt+perlite	25	111•9	(77•9)	595•6	(326•6)
M7	Pt+perlite	3	20•2	← not available →		
M8	Pt+perlite	25	149•1	(125•1)	298•8	(20•6)
M8	Pt+perlite	3	15•2	← not available →		
Control	Pt+perlite	25	124•1	(79•3)	263•3	(0•8)

Key

Pt = Peat

* = One replicate

The above results (Table 12) indicate that there is possibly a fungus effect on the root/shoot ratios which is different for the two substrates. Generally plants inoculated with root isolates and grown in perlite had more root growth and less shoot growth than uninoculated plants. Conversely plants grown in the peat/perlite mixture and inoculated with root isolates had less root growth and more shoot growth than uninoculated plants.

The controlled environment method was of limited success. Ectomycorrhizae occurred on the roots of pine seedlings inoculated with Boletus luteus in both dilute and normal concentrations of the nutrient medium (Fig. 14). All the seedlings (11) in dilute medium were ectomycorrhizal, and at the normal concentration 7 out of the 8 seedlings were ectomycorrhizal.

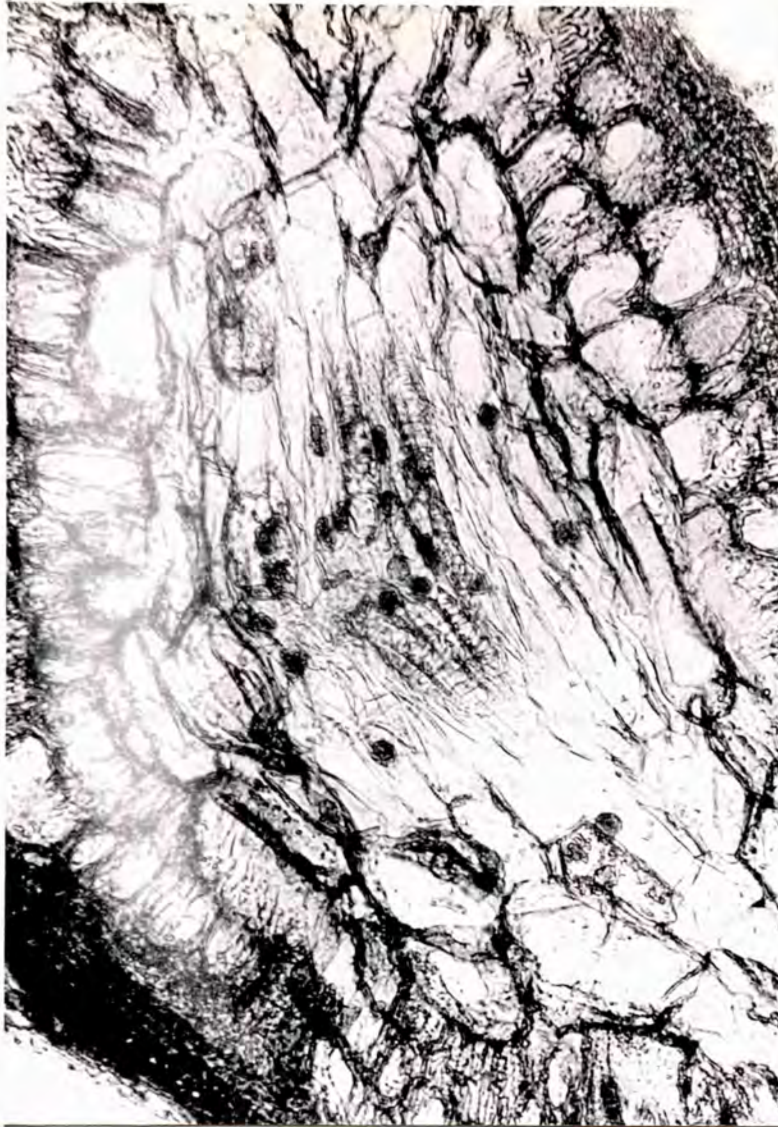
In all flasks (inoculated and uninoculated), the shoots of the seedlings grown in a dilute nutrient medium were slightly chlorotic, even when ectomycorrhizae had formed, whereas those grown in the normal concentration of nutrient medium looked healthy.

The ectomycorrhizal roots obtained with B. luteus showed well developed Hartig net and fungal mantle (see Fig. 15). The ectomycorrhizae which formed on roots grown in dilute nutrient medium were much less abundant than those grown in normal medium. Also in the dilute medium the ectomycorrhizal roots were confined to clusters near the junction of the root and shoot and thus near to the surface of the perlite (Fig. 16).

The fungal growth in all the flasks was concentrated around the roots of the seedlings, this being observed when the plants were removed from the flasks, but since most of the fungi were the same colour as the substrate, the actual density of fungal growth was not obvious.

Magnification x 2•5

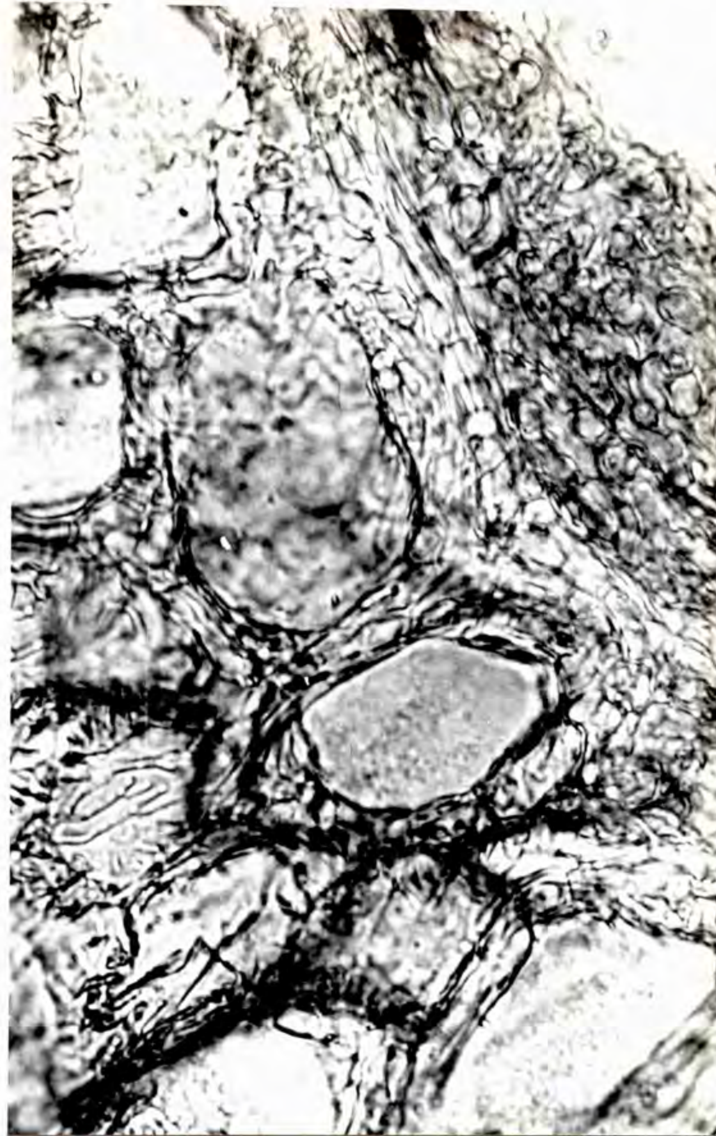
Fig. 14. Controlled environment method for the synthesis of ectomycorrhizae in vitro.
Synthetic ectomycorrhizae formed between Pinus sylvestris seedlings grown in a dilute nutrient medium and Boletus luteus.



Magnification x264

Fig. 15. Controlled environment method for the synthesis of ectomycorrhizae in vitro.
Transverse sections of a synthetic ectomycorrhiza formed between Pinus sylvestris and Boletus luteus to show the details of the mantle and Hartig net.

Continued .....



Magnification x 1256

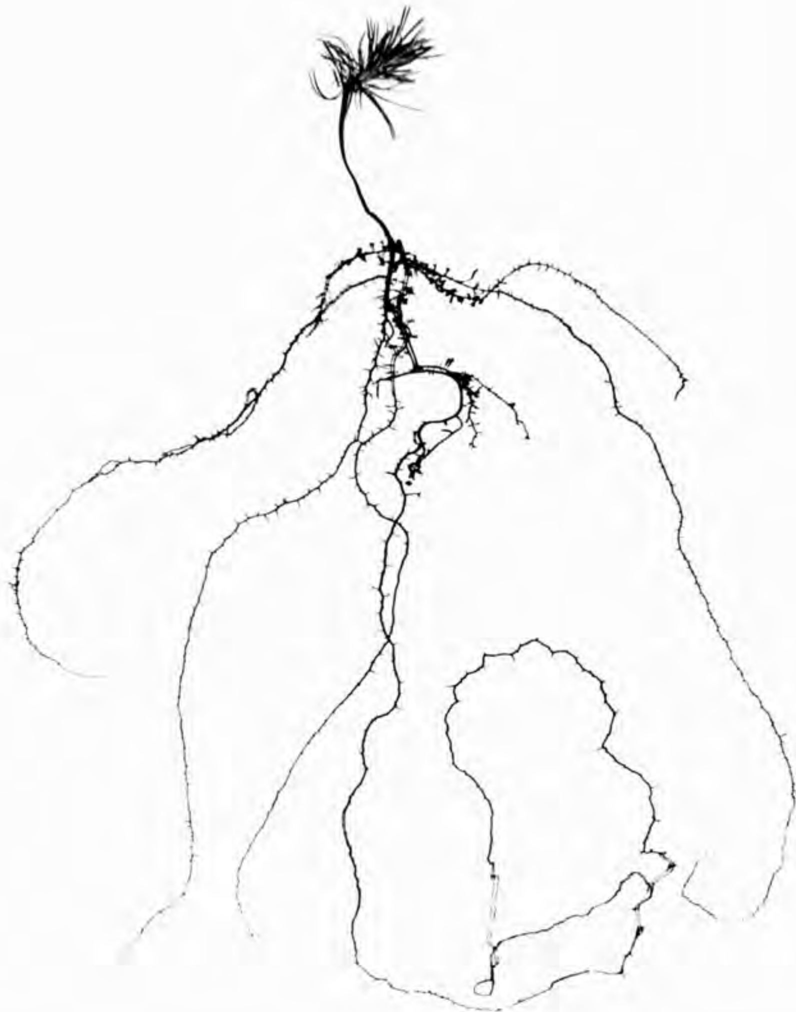
Fig. 15. (cont.)

Magnification x 0•25

a) Seedlings grown in normal nutrient medium.

Fig. 16. Controlled environment method for the synthesis of ectomycorrhizae in vitro.
Synthetic ectomycorrhizae formed between Pinus sylvestris and Boletus luteus.

Continued .....



Magnification x 0·25

b) Seedlings grown in dilute nutrient medium.

Fig. 16. (cont.)

The *in vitro* synthesis of ectomycorrhizas in pots i.e. with their shoots exposed normally to the atmosphere, was also unsuccessful, even though non - sterile humus was collected from sites from which the fungi were origionally isolated. Such humus should have contained potential ectomycorrhizal forming fungi.

Table 13.

Growth of Pinus sylvestris seedlings in sterile and

Fungus	Substrate	Origin	Period of incubation (months)
Control	Non-sterile humus	Thetford	5
M1	Non-sterile humus	Thetford	4
Boletus luteus	Non-sterile humus	Thetford	4
Control	Sterile humus	Thetford	5
M1	Sterile humus	Thetford	8
M23	Sterile humus	Thetford	4
M23	Sterile humus	Thetford	8
Boletus luteus	Sterile humus	Thetford	8
Control	Non-sterile humus	Angley	10
M78	Non-sterile humus	Angley	10
M65	Non-sterile humus	Angley	5
Control	Sterile humus	Angley	5
M13	Sterile humus	Angley	5
M37	Sterile humus	Angley	5
M78	Sterile humus	Angley	5

non-sterile humus inoculated with various root isolates and B. luteus.

Root mean dry weight (mg)	Standard error	Shoot mean dry weight (mg)	Standard error	Number of replicates
111•2	(68•2)	327•6	(172•6)	3
52•2	(33•2)	163•3	(105•9)	5
48•9	(18•3)	169•6	(81•8)	6
135•6	(35•6)	557•0	(141•6)	4
358•0	(71•4)	1416•3	(101•8)	4
157•7	(27•6)	510•5	(6•9)	2
191•7	(143•0)	763•8	(682•9)	3
263•9	(118•0)	933•6	(304•3)	5
86•5	(55•2)	238•8	(187•5)	4
154•5	-	368•5	-	1
227•8	-	500•2	-	1
139•7	(87•8)	506•4	(228•8)	4
87•5	(36•1)	408•3	(227•7)	4
179•1	(55•7)	577•8	(168•6)	9
116•5	(64•6)	532•6	(292•9)	6

The growth of the control seedlings in non-sterile humus (see Table 13 above), was not as great as those grown in sterile humus. Clearly autoclaving the soil did not have any deterimental effect on the growth of the pine seedlings. Also under these conditions inoculation of the humus did not influence the seedling growth in a regular and clear cut way as was found with those seedlings grown in the "Melin flasks" (see page 141 and Table 12). However it should be noted that different root isolates were used (except M1).

THE EFFECT OF INDOLE - 3 - ACETIC ACID (IAA) ON THE DEVELOPMENT OF PINUS SYLVESTRIS ROOT SYSTEMS.

Observations of the tubes over the duration of this experiment i.e. nine months, showed that IAA at the higher concentrations, 10^{-4} to 10^{-5}M, added either initially or after a period of 4 weeks to the growth tubes, caused inhibition of root growth. The main root became swollen, also the lateral roots were very swollen and distorted, and eventually these seedlings died. At a concentration of 10^{-6}M again the main root growth was greatly retarded and this root became very much swollen, but the lateral roots showed none of the swelling of the main root. This occurred when IAA was added initially to the tubes, but when added to the seedlings after 4 weeks growth there was no stunting of the main root, except at the tip, and the secondary laterals divided dichotomously to produce structures (see Fig. 17) very similar to those produced by pine seedling roots in the presence of M1, M3, M7, Boletus luteus and Boletus variegatus 1 (see page 138 and Fig. 13).

At the lower concentrations of IAA i.e. 10^{-7} and 10^{-8}M, added either immediately or after 4 weeks to the tubes, the root growth was very similar to that of the control seedlings, the main root showing no signs of any swelling and only a few short primary lateral roots being produced. At 10^{-9} to10^{-11}M, the main root of the seedlings grew approximately twice as long as the control roots, although the lateral root formation was similar to that of the control.

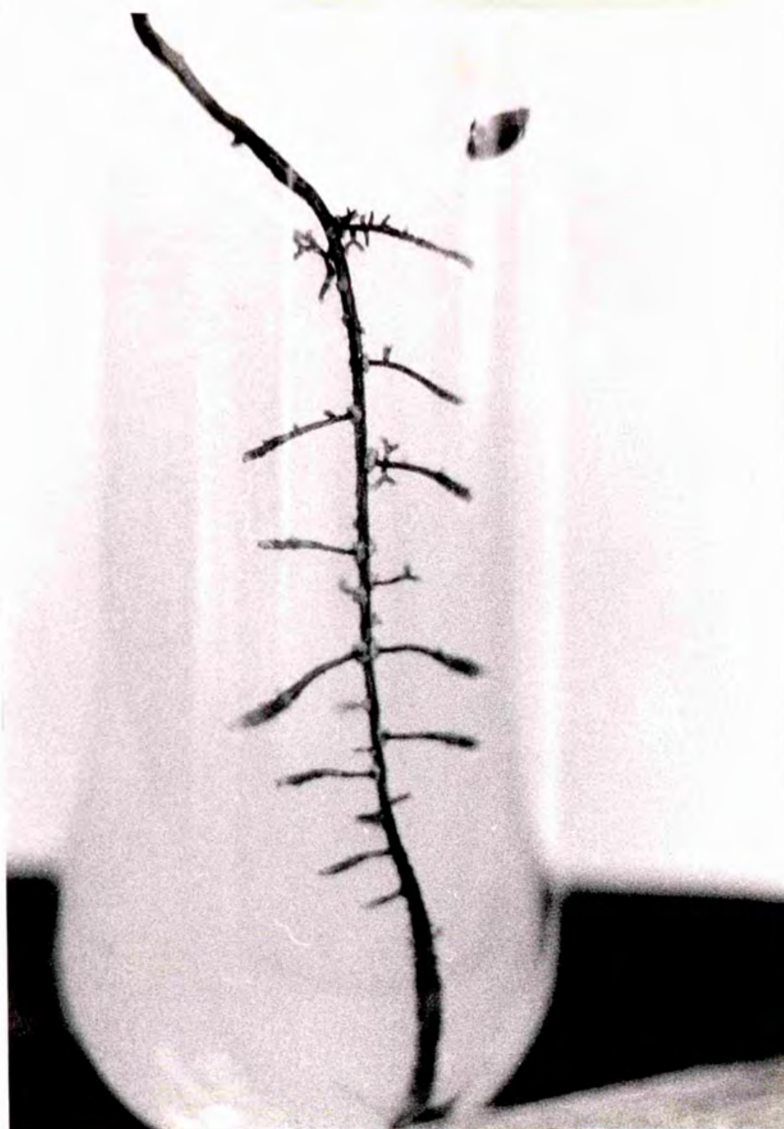
After nine months the pine seedlings remaining in the tubes were removed, washed, and then dried for 48 hours at 70°C, to determine their final dry weights. The mean dry weights are given in the following table (Table 14).

Magnification x 2·5

a) No IAA (control).

Fig. 17. Dichotomous secondary lateral roots produced after four months growth of Pinus sylvestris seedlings in the presence of 10^{-6}M IAA.

Continued .....



Magnification x 2·5

b) 10^{-6}M IAA added after four weeks growth.

Fig. 17. (cont.)

NUTRITIONAL EXPERIMENTS.

Growth in BLM and MLM and in mineral solution.

The growth of three root isolates, M1, M3, and M13 in BLM and MLM is shown in Table 15.

Table 15.

The growth of M1, M3, and M13 in BLM and MLM.

Root isolate	Growth after 14 days (dry weight of mycelium in mg)				P (Increase in dry weight of root isolate in MLM compared with BLM)
	BLM	Standard error	MLM	Standard error	
M1	167•0	(12•0)	236•2	(41•6)	0•001
M3	224•8	(32•3)	256•4	(70•4)	n.s.
M13	60•6	(11•0)	141•3	(3•2)	0•001

The effect of adding amino acids and vitamins to BLM varies with the isolate. Growth curves in BLM and MLM, over a period of 26 days, are given for M1 and M3 in Fig. 18. These emphasize the different response of these two isolates to MLM and the effect is confirmed by their growth on BIA and MIA (Fig. 19).

The effect of various components of the MLM on the growth of M13 is given in Fig. 20. This isolate shows a nearly complete requirement for thiamin. In the presence of thiamin M13 responds positively to the amino acid mixture used in MLM, but not significantly to the additional vitamins. However when the amino acids and vitamins are added together i.e. as MLM, there is a slight positive interaction.

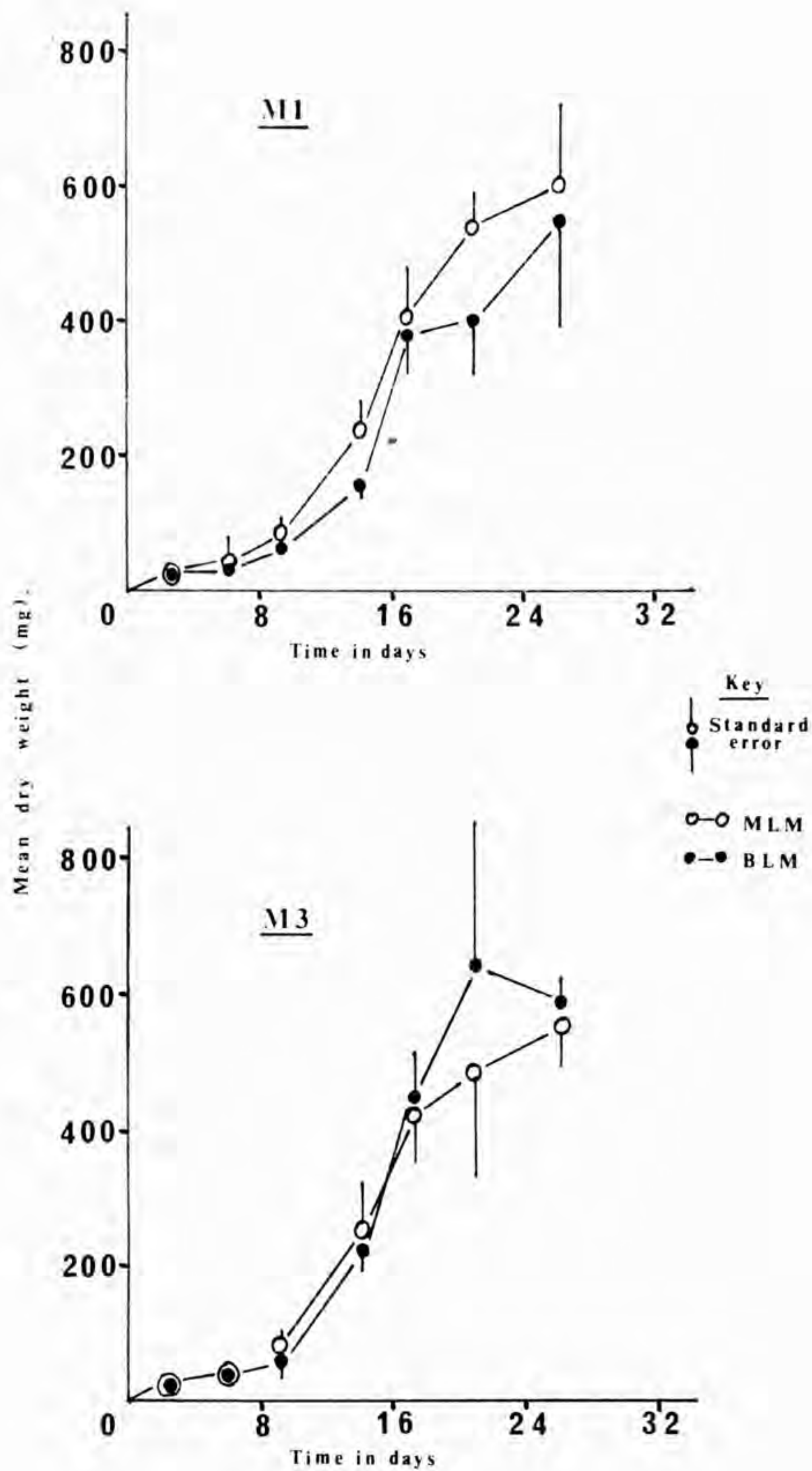


Fig. 18. The growth of M1 and M3 in BLM and MLM.

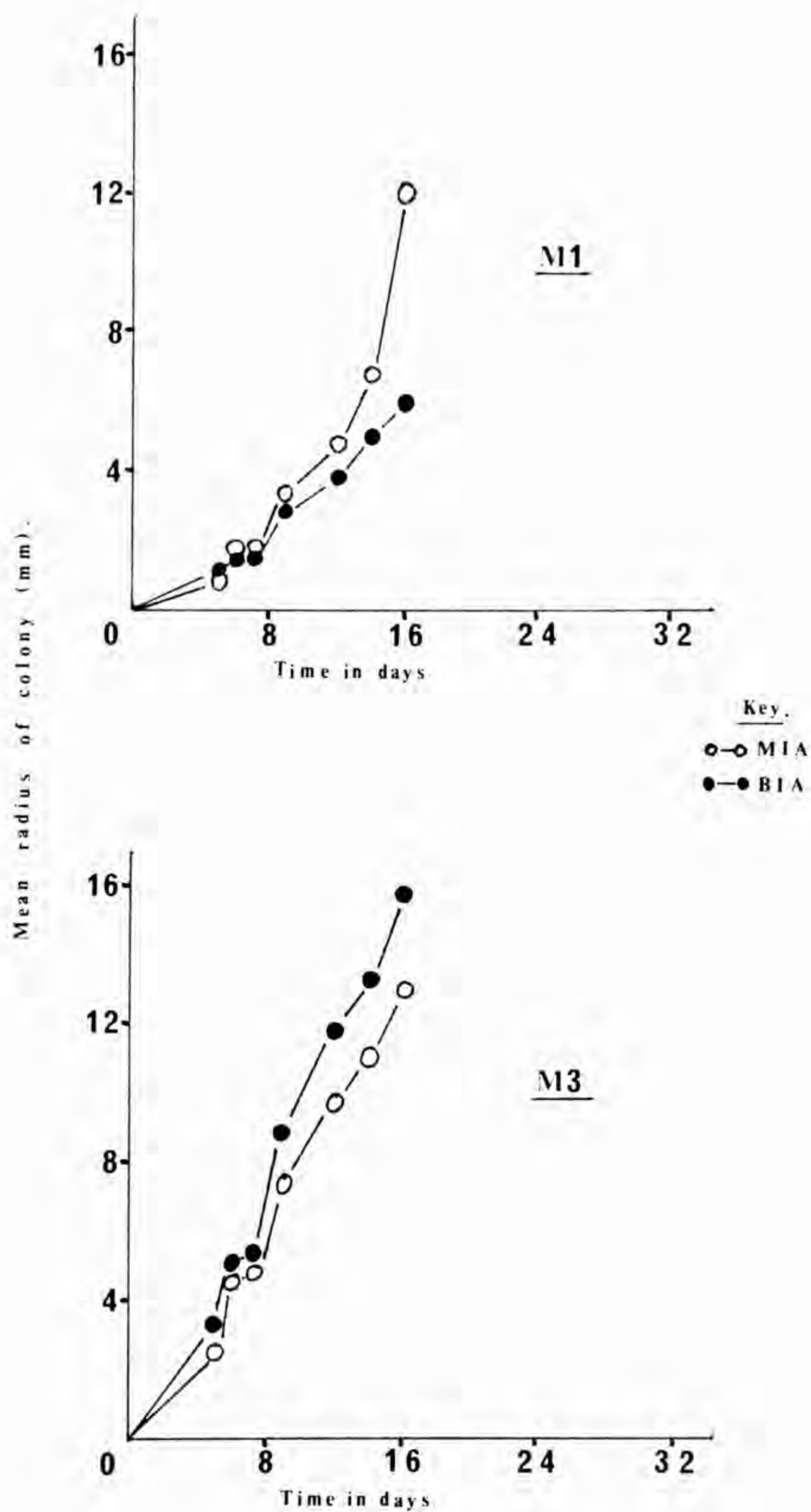


Fig. 19. The growth of M1 and M3 on BIA and MIA.

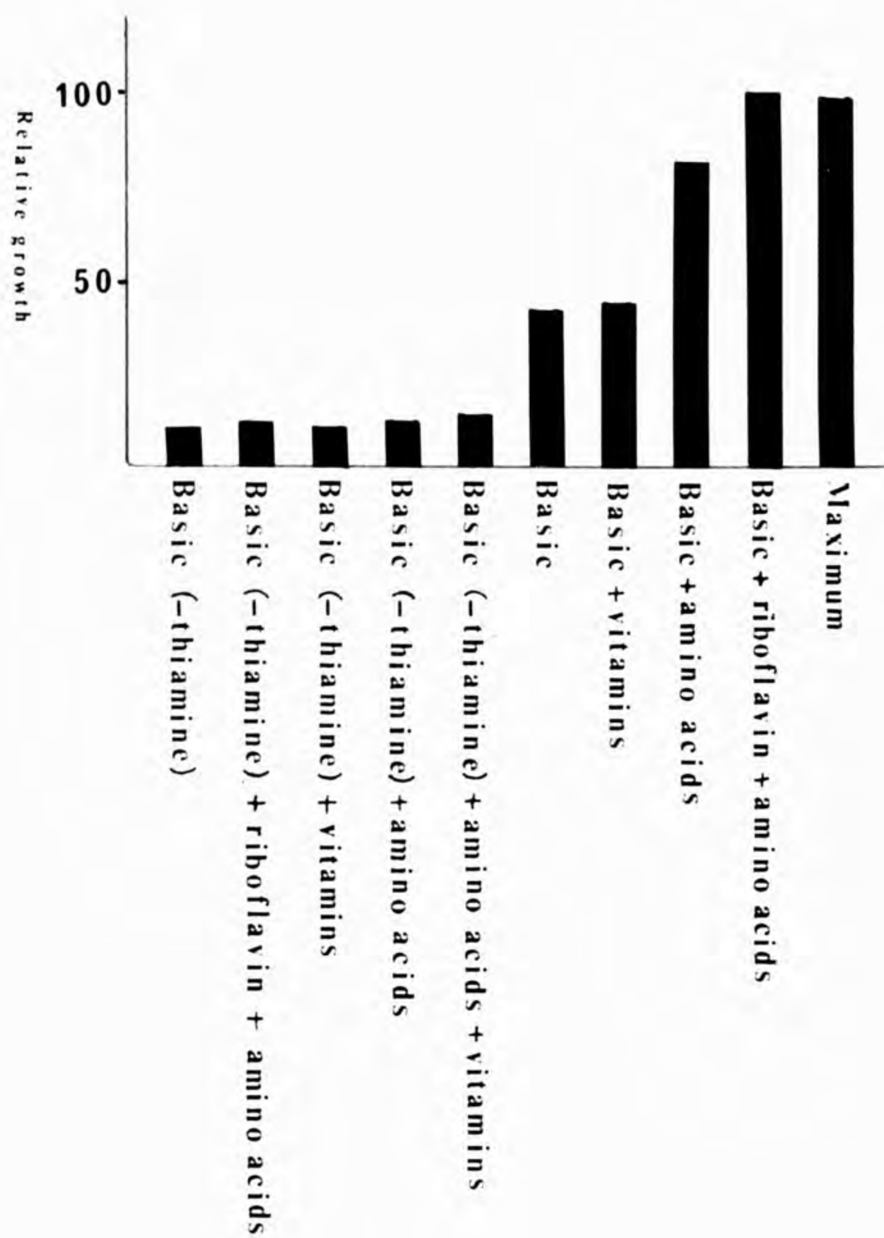


Fig. 20. Growth of M13 with amino acids and vitamins.

The optimum temperature for the growth of M13 on MIA was 20°C as shown in Fig. 21.

The optimum pH for this fungus would appear to be at the lower end of the range of buffer systems used i.e. about pH 2•2 as indicated in Fig. 22.

The growth of M13 with various nitrogen sources in BLM is given in Table 16.

Table 16.

Growth of M13 with various nitrogen sources.

Nitrogen source	Mean dry weight, mg	Standard error	P (Increase in growth over NH_4 tartrate)
NH_4 tartrate	26•5	(1•2)	-
KNO_3	33•3	(4•8)	0•02
Peptone	34•0	(4•7)	0•02

Growth was slightly better on peptone and potassium nitrate than on ammonium tartrate.

The growth of M13 with various carbon sources is shown in Table 17 and Fig. 23.

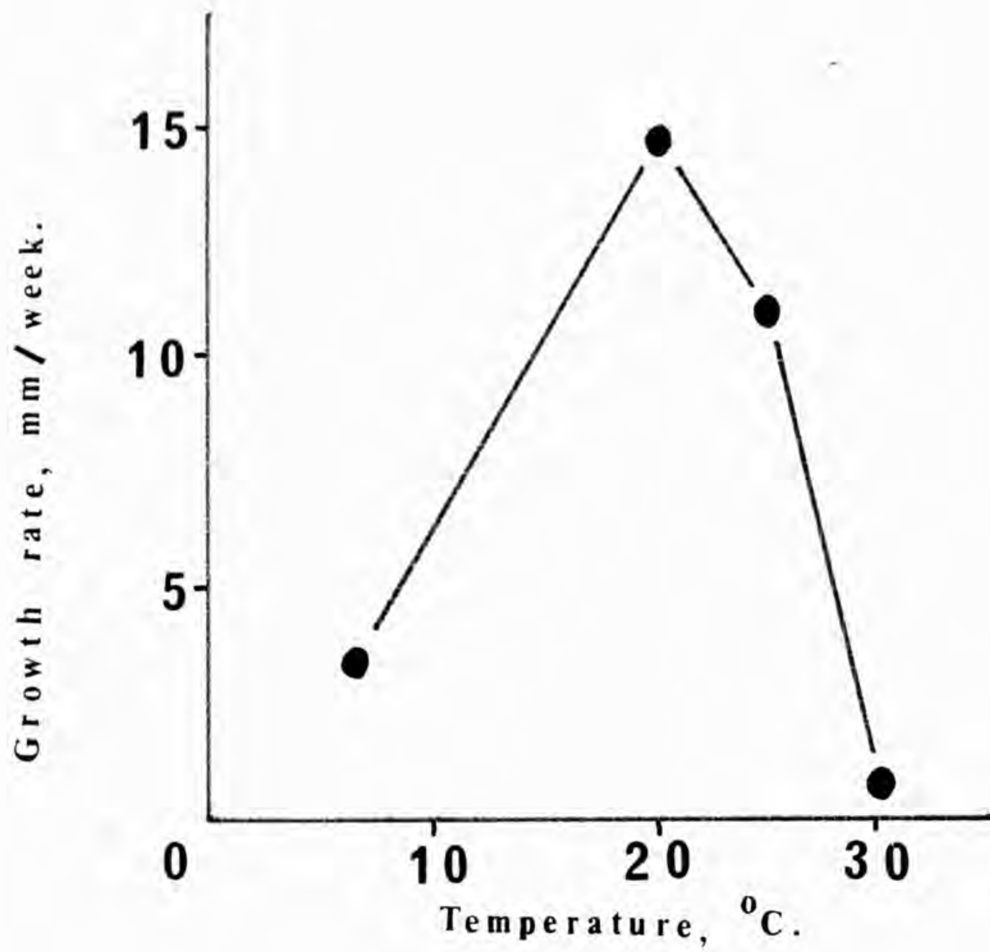
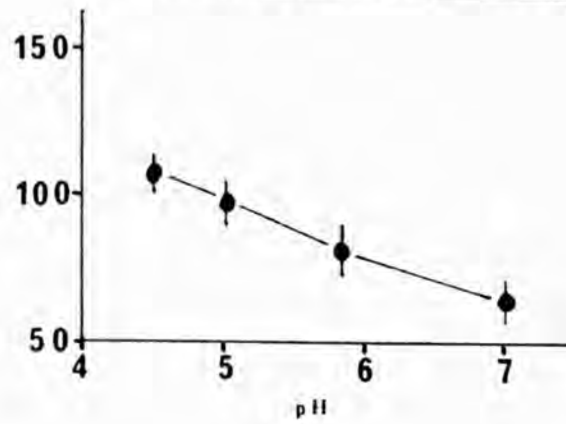
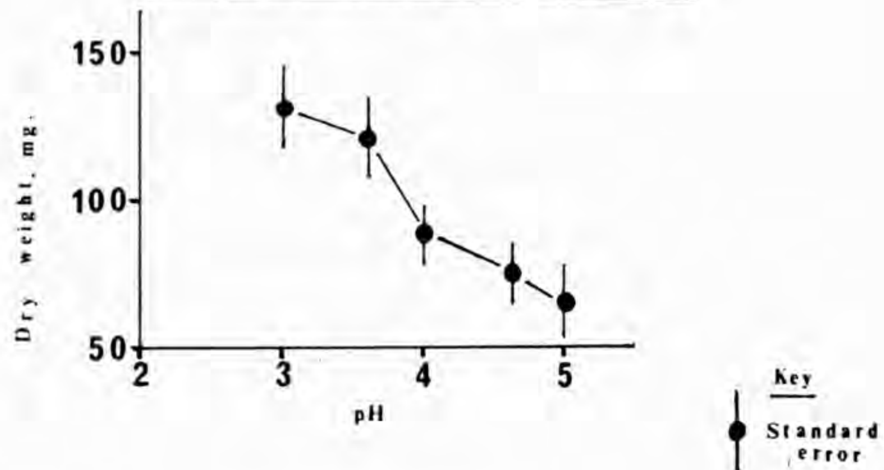


Fig. 21. Temperature growth curve for M13





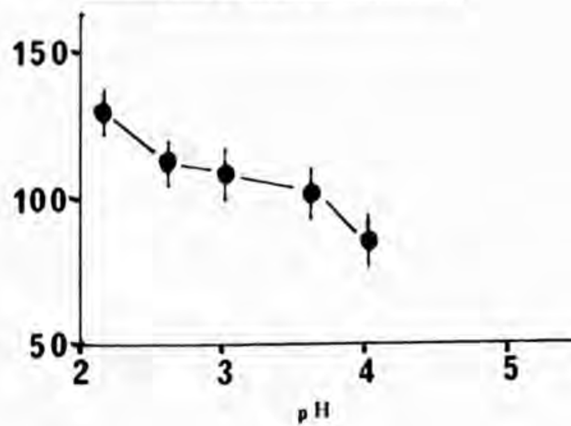


Fig. 22. pH growth curves for M13.

Table 17.

Growth of M13 with various carbon sources in BLM.

Carbon source (2% sugar)	Mean dry weight, mg	Standard error	P
None	1•1	(0•5)	0•001 (inhibition)
Glucose (control)	69•2	(8•3)	-
Galactose	86•7	(10•7)	0•02
Fructose	86•2	(20•2)	n.s.
Sucrose	89•1	(6•9)	0•01
Mannose	83•2	(10•0)	0•05
Mannitol	93•5	(9•3)	0•01
Maltose	89•5	(15•1)	0•05
Acetate	0•0	(0•0)	Inhibition
Malate	37•2	(16•1)	0•01 (inhibition)
Xylose	111•3	(7•7)	0•001

Xylose gave the best growth and galactose, sucrose, mannose, mannitol and maltose all gave more growth than glucose. Malate produced poor growth and acetate was completely inhibitory. Some growth did occur in the flasks containing no carbon source, this possibly due to the carry-over of glucose in the inoculum from the BLM in which it was origionally grown.

The ability of M13 to utilize various polysaccharides in BLM with and without the addition of a trace (0•05%) of glucose is shown in Table 18 and Fig. 24.

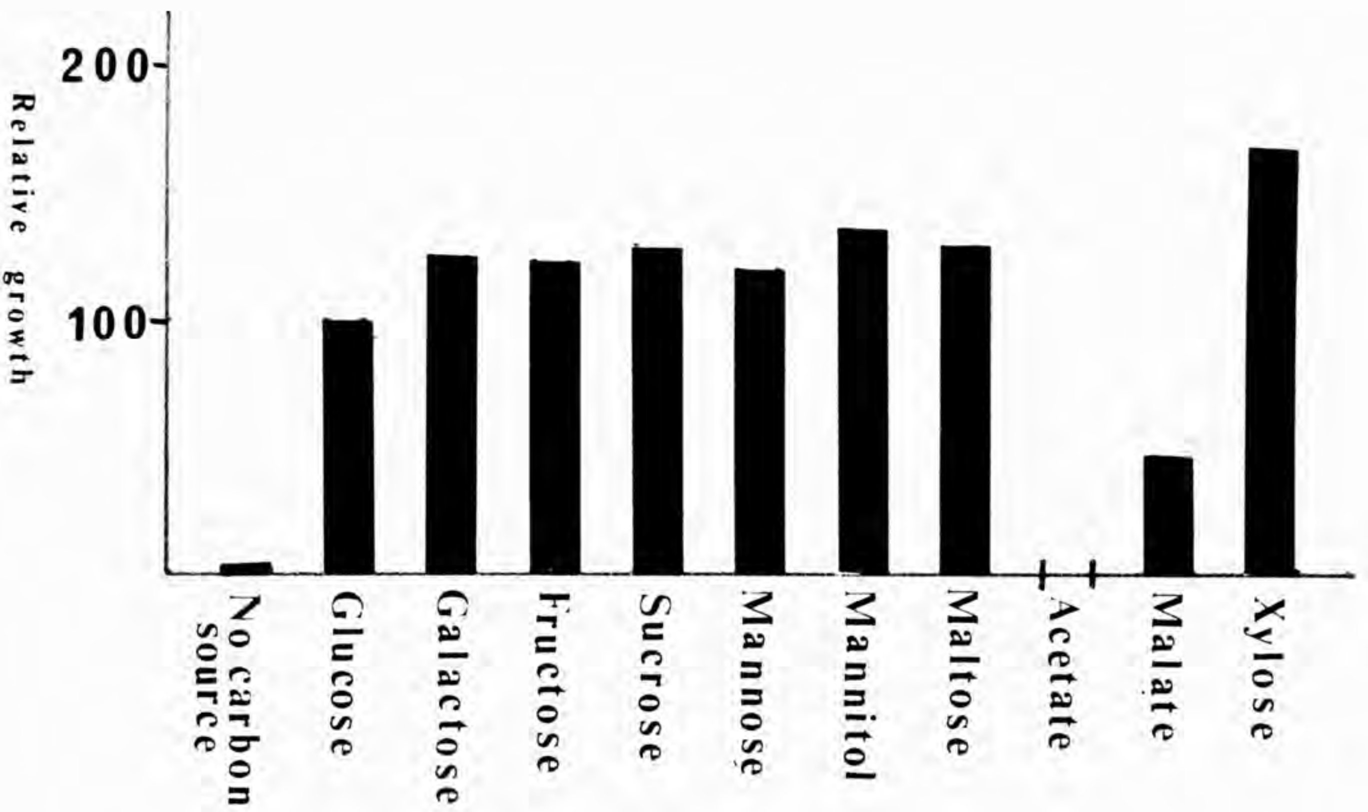


Fig. 23. Growth of M13 with various carbon sources in BLM.

Table 18.

Growth of M13 with various polysaccharides in BLM.

Carbon source	Mean dry weight, mg	Standard error	*p	Interaction with 0•05% glucose
None	1•9	(2•1)	-	-
Glucose (2%)	66•5	(8•5)	-	-
Glucose (0•05%)	57•7	(6•9)	-	-
Polypectate (1%)	70•8	(18•0)	-	-
Polypectate (1%)+glucose(0•05%)	85•7	(18•9)	0•001	Negative
CMC (1%)	0•6	(0•2)	-	-
CMC (1%)+glucose (0•05%)	91•3	(8•7)	0•05	Positive
Starch (2%)	89•9	(12•8)	-	-
Starch (2%)+glucose (0•05%)	105•1	(6•1)	0•001	Negative
Pectin (1%)	48•8	(2•7)	-	-
Pectin (1%)+glucose (0•05%)	47•8	(4•1)	0•001	Negative

* Significance determined by the *t* distribution.

Starch gave more growth than glucose. Growth with polypectate at 1% was very similar to that obtained with glucose at 2%, whereas pectin and CMC (both at 1%) gave less growth than glucose at 2%.

The presence of a trace of glucose had little effect on the ability of M13 to utilize these polysaccharides except CMC, where there was a significant positive interaction.

The results of experiments to determine whether selected root isolates are able to utilize cellulose and lignin in pinewood sawdust are given in Table 19 and Fig. 26.

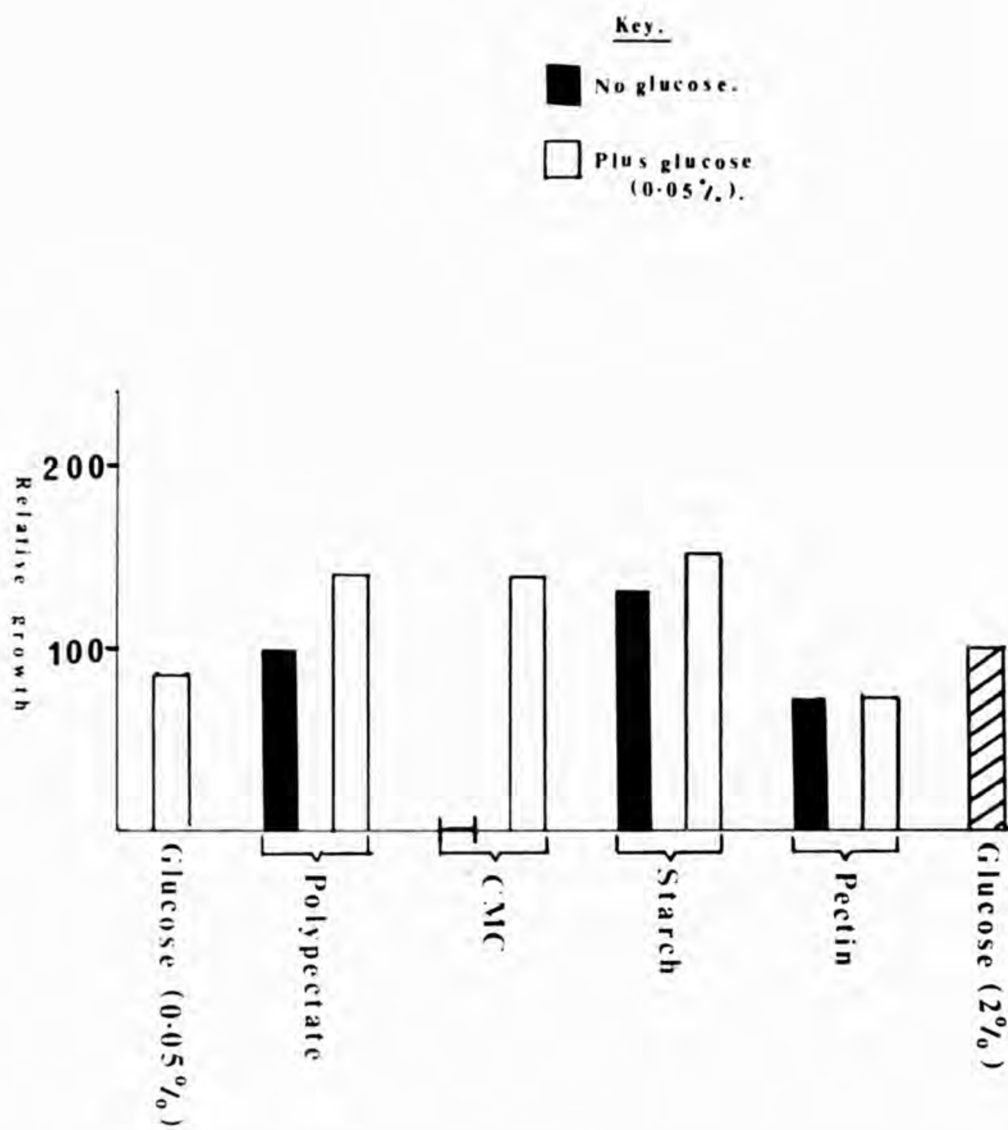


Fig. 24. Growth of M13 with various polysaccharides, in BLM.

Table 19.

The amount of lignin and cellulose decomposed by four

Fungus	Dry weight of sample used in analysis, mg	*Mean dry weight of lignin, mg. (As lignin-thioglycolic acid).	Standard error	F	A Lignin decomposed, %
None (control)	348•8	87•8	(11•6)	-	-
M13	353•7	74•8	(16•3)	n.s.	14•8
M13+glucose (0•1%)	343•2	72•0	(4•1)	0•05	18•0
M23	332•9	72•7	(3•7)	0•05	17•2
M23+glucose (0•1%)	364•4	83•5	(8•8)	n.s.	4•9
M37	346•3	76•3	(7•3)	n.s.	13•1
M37+glucose (0•1%)	363•4	74•6	(10•5)	n.s.	15•0
M65	348•8	87•8	(4•0)	n.s.	0•0
M65+glucose (0•1%)	353•7	98•3	(8•4)	n.s.	12•0 (increase)

* Includes the correction value (see Fig. 25)

root isolates in pinewood[†] sawdust after six months growth.

Mean dry weight of cellulose, mg	Standard error	P	B cellulose decomposed, %	Quotient B/A
133•8	(48•2)	-	-	-
94•2	(1•1)	n.s.	29•6	2•0
85•2	(4•7)	0•05	36•3	2•0
102•1	(10•9)	n.s.	23•7	1•3
95•8	(10•4)	n.s.	28•4	5•8
122•1	(7•3)	n.s.	8•7	0•7
117•1	(22•8)	n.s.	12•5	0•8
142•7	(20•9)	n.s.	6•7 (increase)	-
117•4	(22•4)	n.s.	12•3	-

† Dry matter per flask before decay = 5•0g

Initial composition of sawdust = cellulose 38•4% and lignin 25•2%

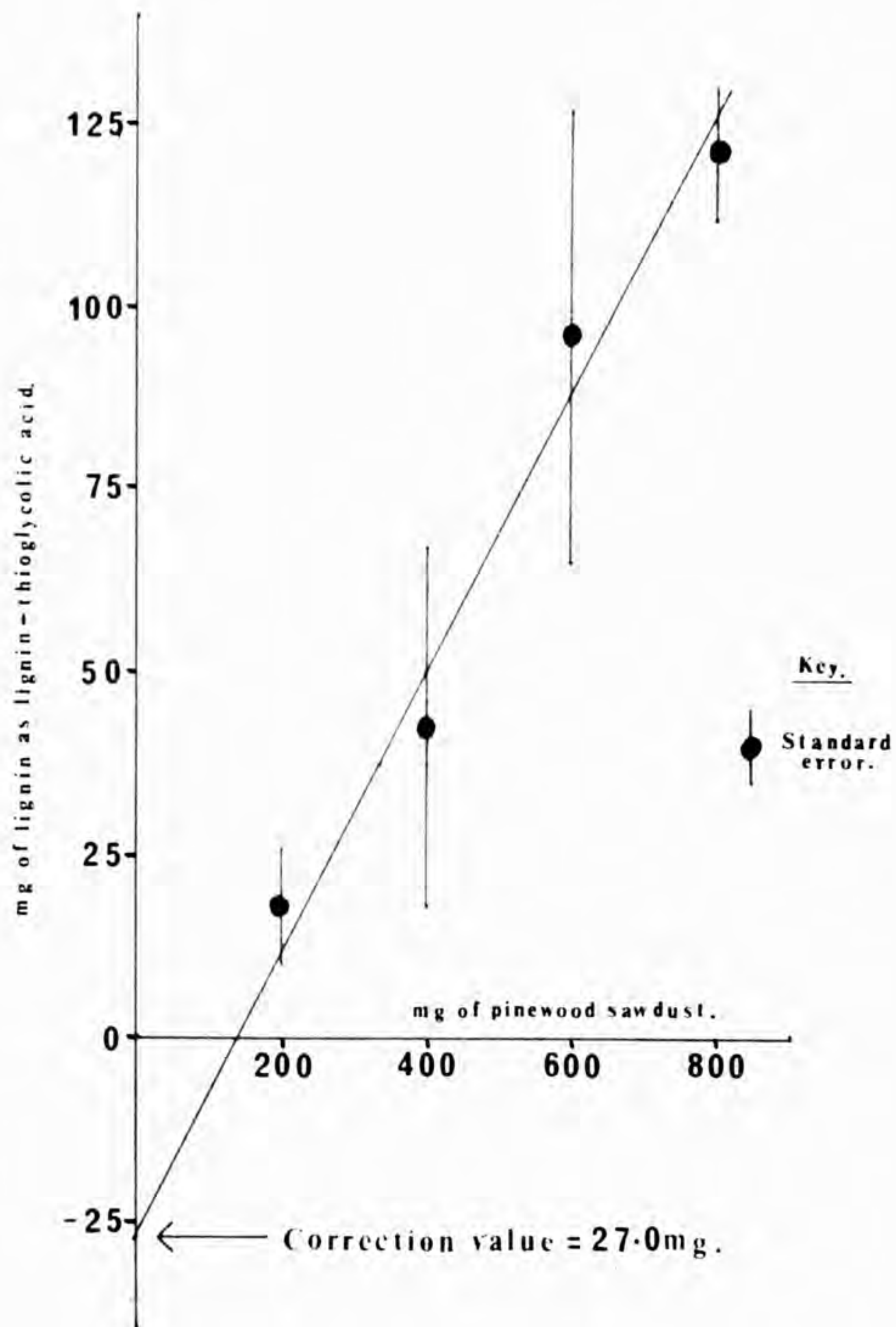


Fig. 25. Correction curve for lignin analysis.

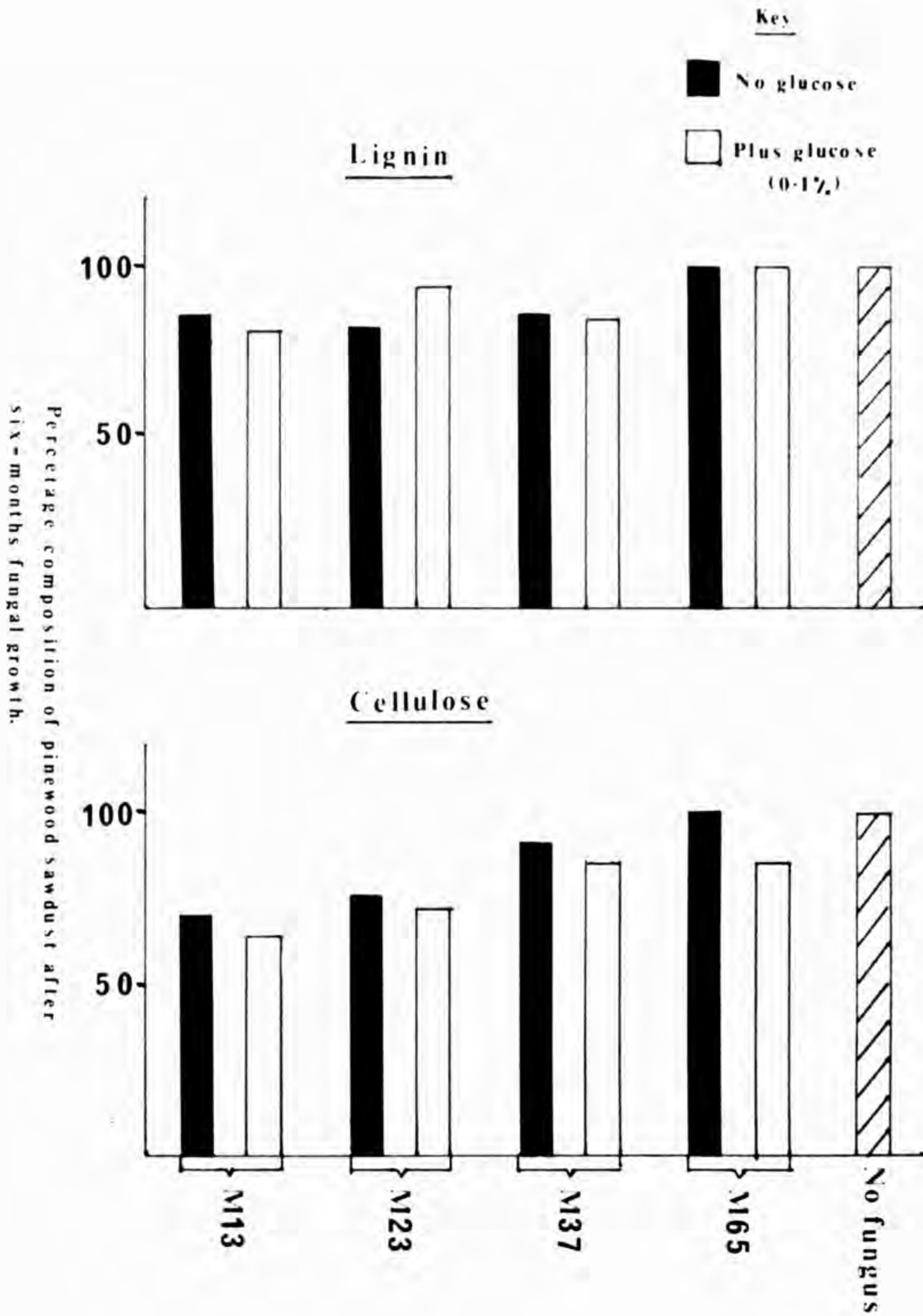


Fig.26. The amount of lignin and cellulose decomposed by four root isolates, in pinewood sawdust, after six-months growth.

M13 showed a significant ability to utilize wood cellulose only in the presence of a trace quantity (0•1%) of glucose. This pinewood cellulose was not decomposed by either M23, M37 or M65.

M23 showed a limited capacity to utilize lignin, whilst M13, M37, and M65 showed none. The addition of 0•1% glucose to the medium had no effect, except to cause adaptive growth with M13, but in the case of M23 lignin utilization was much reduced.

The ability of the above four isolates to grow on ground up filter paper, added to a medium of BIA less glucose is shown in Table 20.

Table 20.

Growth of root isolates on BIA plus cellulose and minus glucose.

Fungus	Mean radius of colony (mm)	Standard error
M13	7•5	(0•0)
M23	17•5	(0•0)
M37	5•0	(0•0)
M65	6•3	(1•8)

All isolates appeared to be able to grow on the cellulose to some extent, M23 being able to utilize the cellulose more than M13, M37 or M65.

EXPERIMENTS WITH EXCISED TOMATO ROOTS.

1. Experiments with live and killed excised tomato roots.

a) Growth of various isolates in the presence of excised tomato roots in MLM.

Over a period of 7 days most of the root isolates and Boletus variegatus 1 were stimulated to some extent by the presence of live excised tomato roots in MLM, except for M2, M5, M8, M10, M22, M65 and M70.

In most cases (see Appendix C and Fig. 27), killed excised tomato roots had no effect on the growth of the isolates after 7 days incubation, but M3, M4, M5, M13, M16 (with 14 day old excised tomato roots), M23, M48 and M78 were all stimulated by them. One isolate, M5, was stimulated only by killed and not by live excised tomato roots.

The root isolates grown in the presence of both live and killed excised tomato roots for 14 days generally show increased stimulation in the presence of roots. Two isolates i.e. M65 and M70 which were not significantly stimulated after 7 days exhibited a significant stimulation ($P = 0{\cdot}001$ and $0{\cdot}05$ respectively), after 14 days, but M78 and B. variegatus 1 showed reduced stimulation after 14 days.

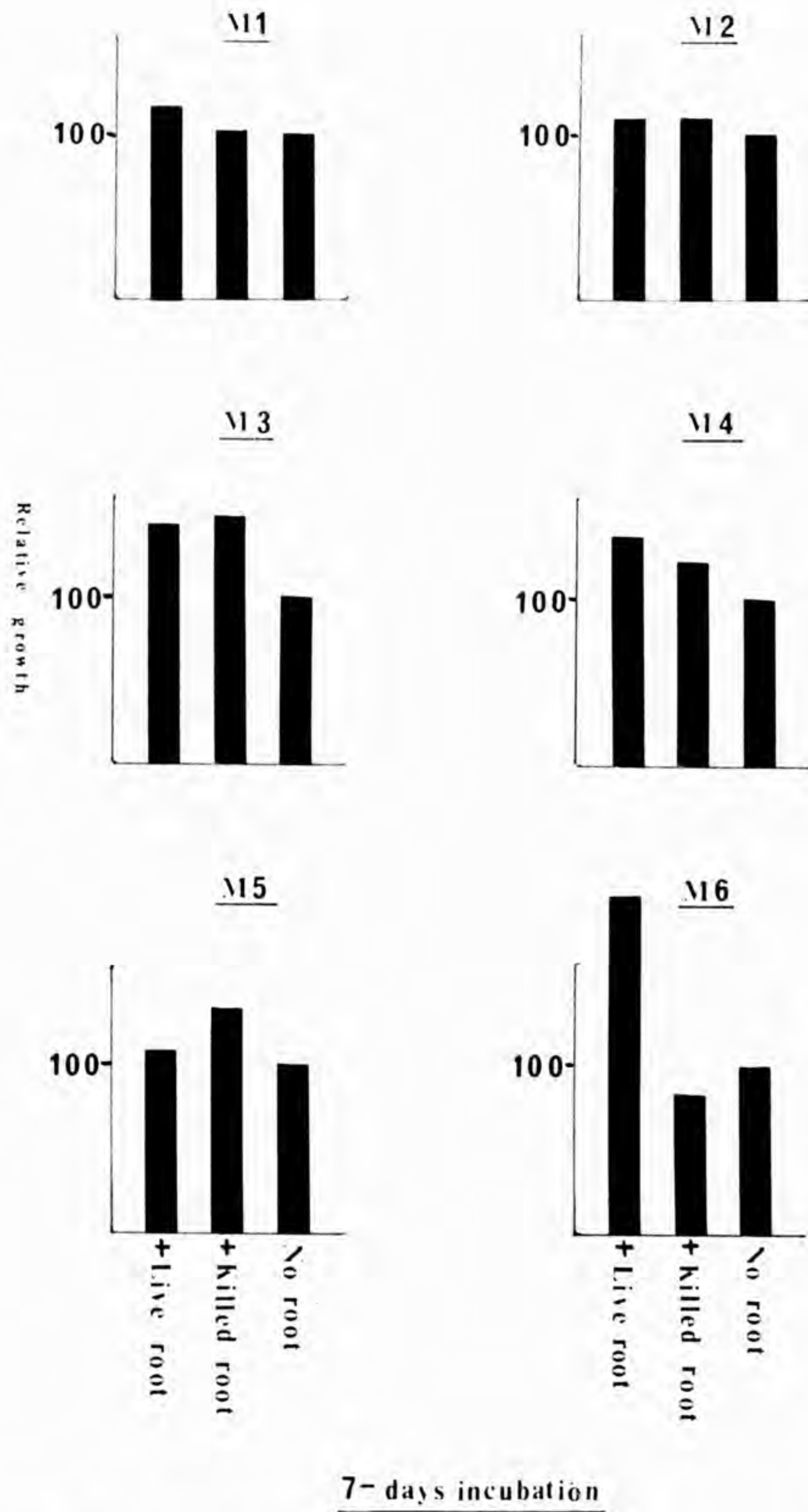


Fig. 27. Growth of root isolates and Boletus variegatus 1 with live and killed excised tomato roots in MLM.

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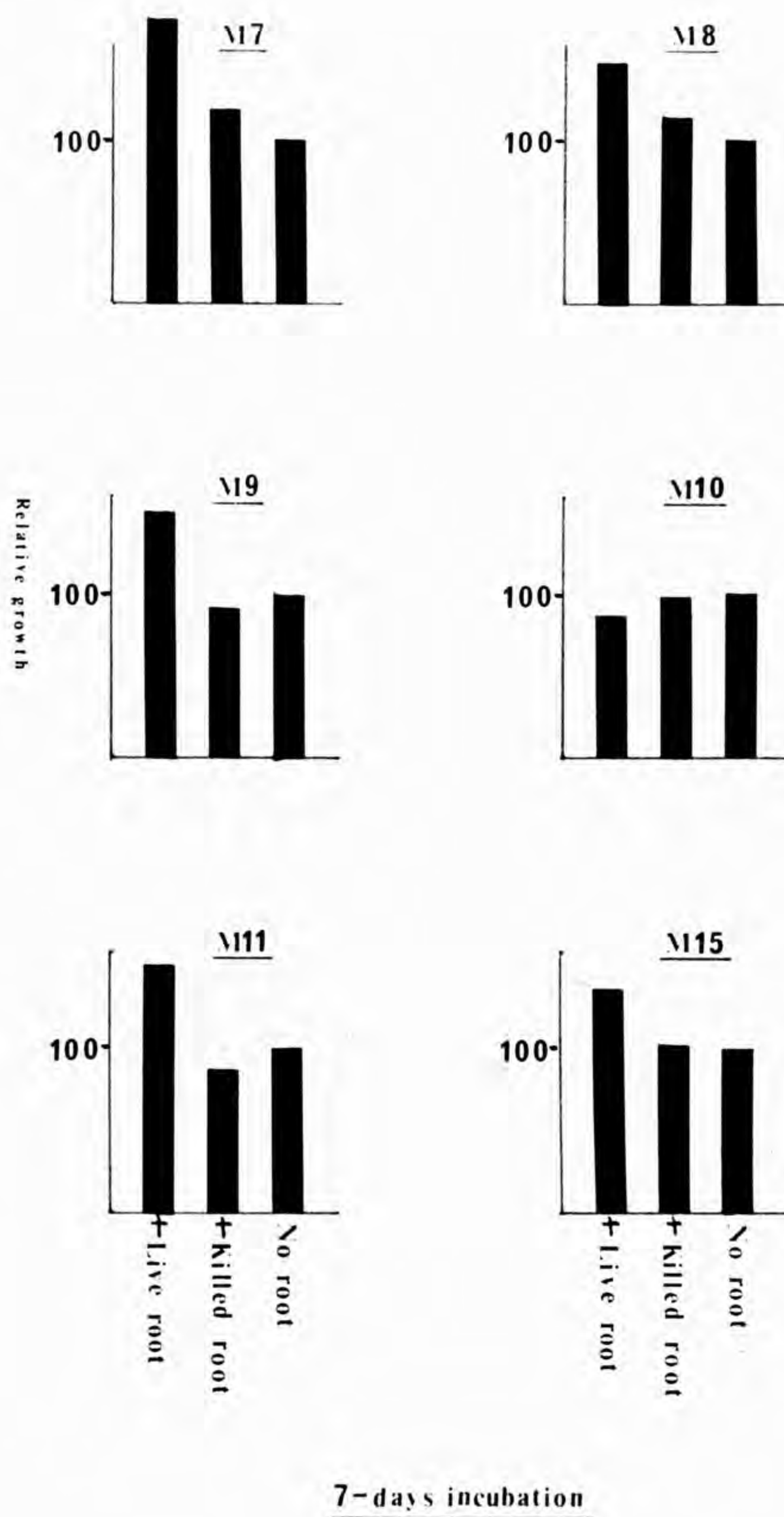

Fig. 27. (cont.)

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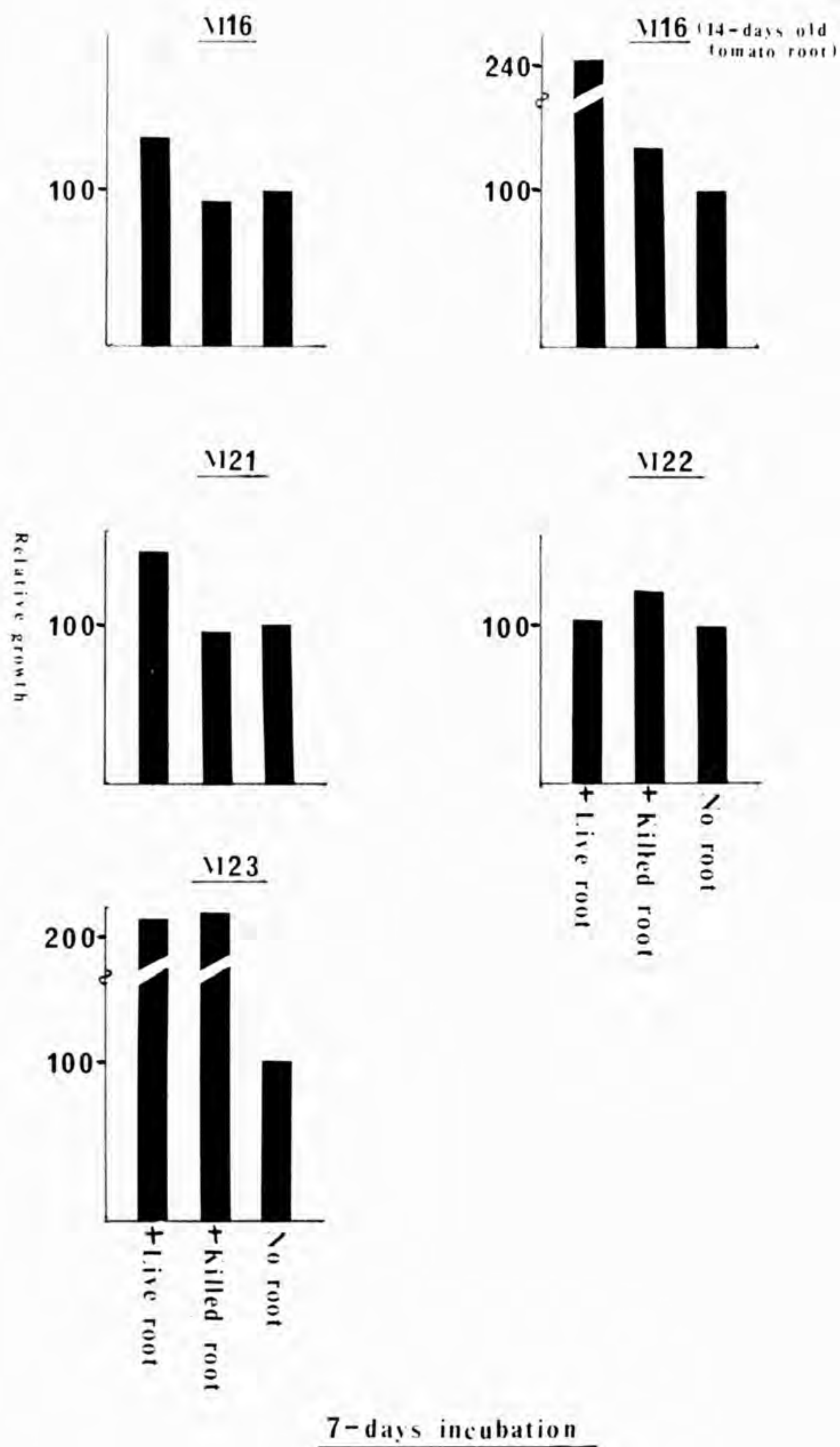

Fig. 27. (cont.)

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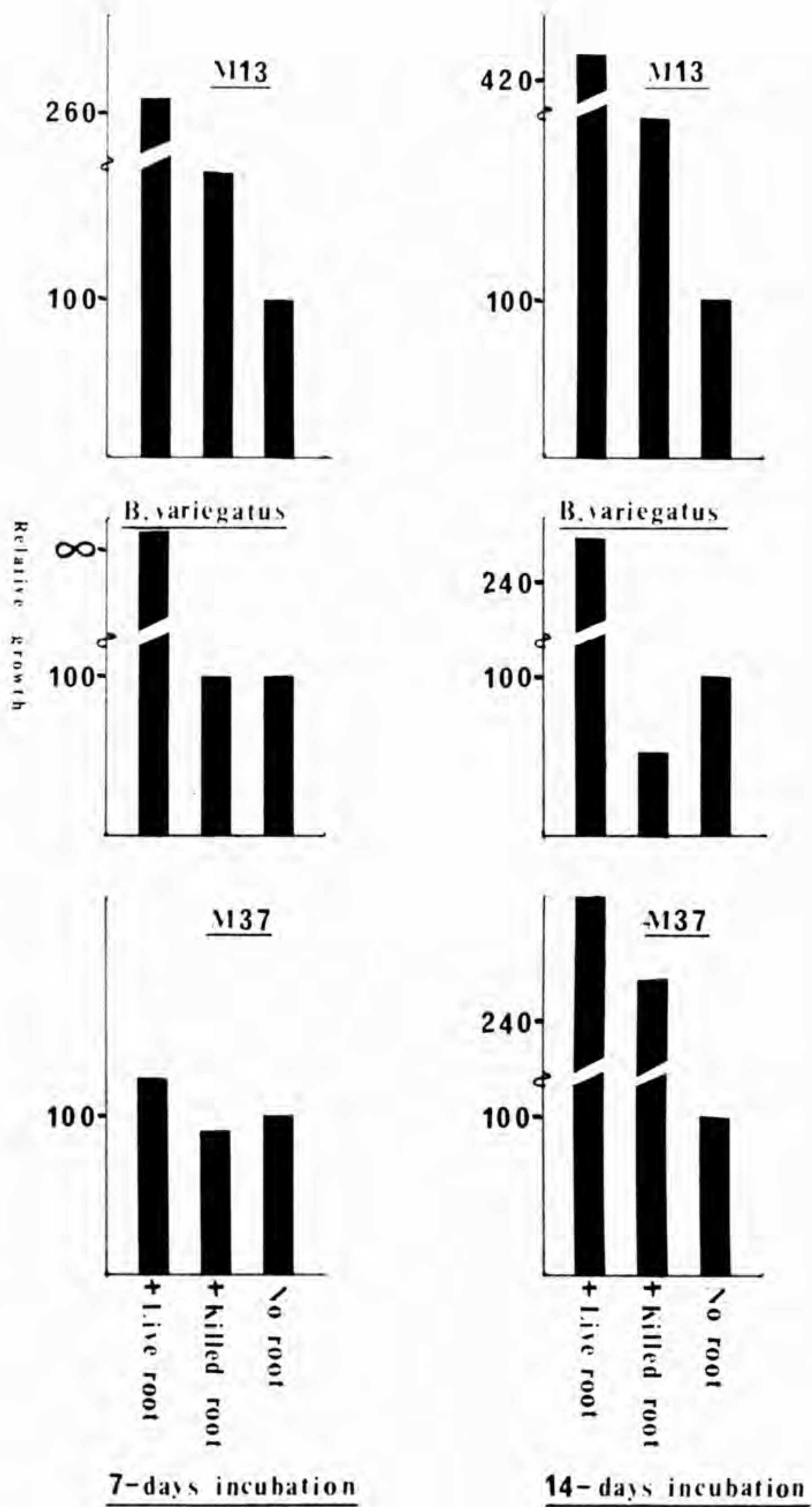

Fig. 27. (cont.)

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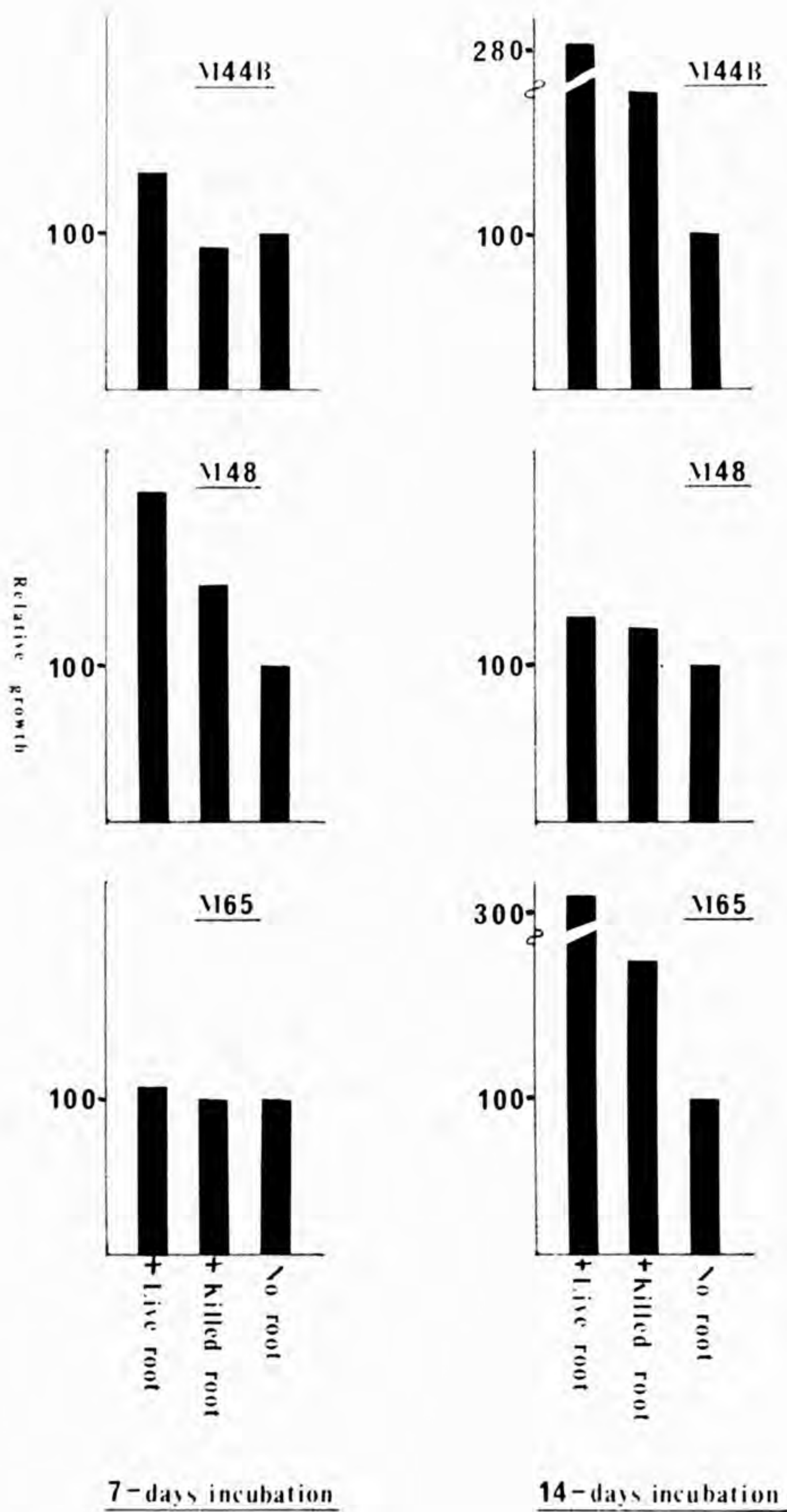

Fig. 27. (cont.)

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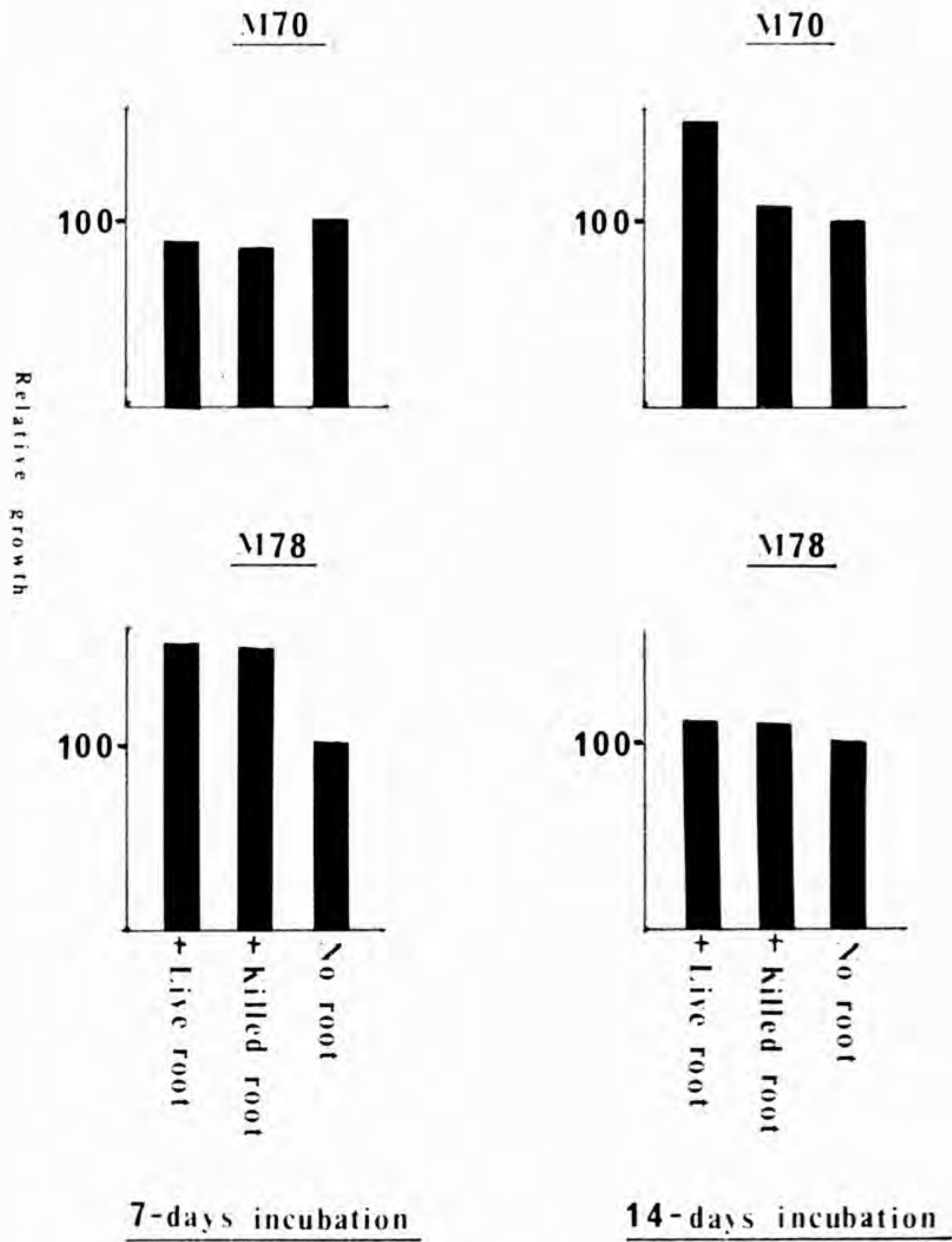


Fig. 27. (cont.)

b) <u>Seventeen-day time course experiment with M13 in MLM.</u>

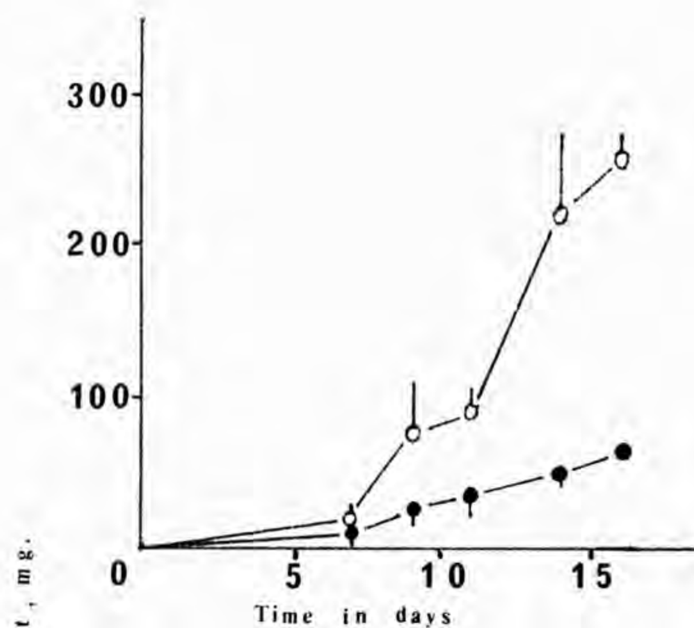
The growth of M13 in MLM and in the presence of live and killed excised tomato roots over a period of 17 days is shown in Fig. 28.

From Fig. 28, the excised tomato roots, both live and killed, as expected, stimulated the growth of M13 over a period of 17 days. The response to the excised live roots was generally about double that to killed roots.

c) <u>Growth of M13 and M23 in the presence of excised tomato roots in BLM and MLM for 14 days.</u>

When M13 and M23 were grown in BLM and MLM, stimulation by both live and killed excised tomato roots was greater in BLM (see Table 21 and Fig. 29).

Live excised tomato root



Mean dry weight, mg.

Killed excised tomato root

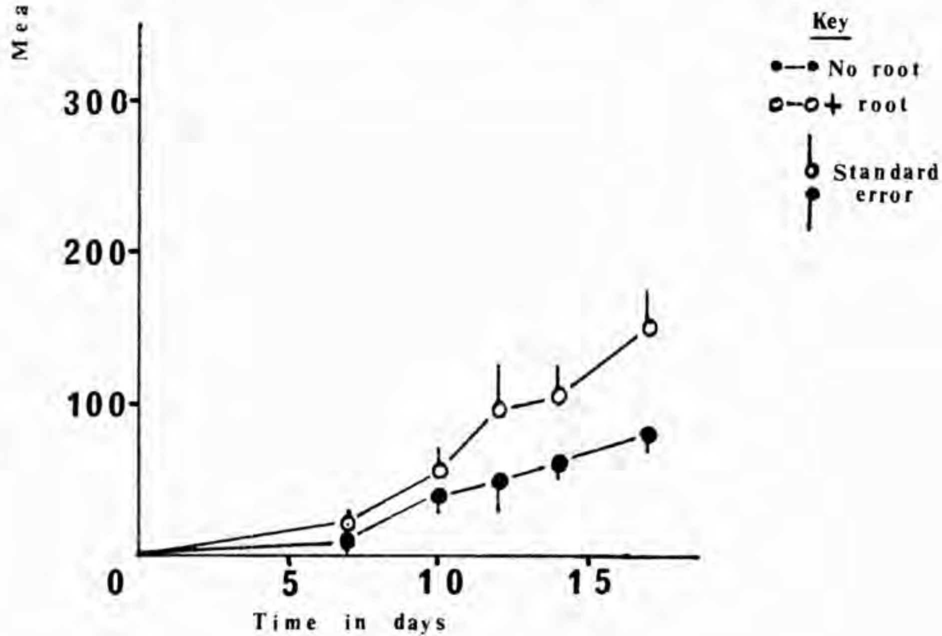


Fig. 28. Growth of M13 in the presence of live and killed excised tomato roots, in MLM.

Table 21.

Growth of M13 and M23 in the presence of excised tomato roots in BLM and MLM for 14 days.

Fungus	Medium	Treatment	*Mean dry weight, mg	Standard error	P
M13	BLM	No root	35•4	(5•4)	-
M13	BLM	+Live root	68•5	(12•4)	0•001
M13	BLM	+Killed root	54•5	(8•7)	0•001
M13	MLM	No root	63•6	(15•6)	-
M13	MLM	+Live root	87•0	(12•4)	0•002
M13	MLM	+Killed root	70•2	(23•2)	n.s.
M23	BLM	No root	51•1	(10•6)	-
M23	BLM	+Live root	82•0	(19•6)	0•001
M23	BLM	+Killed root	79•0	(8. 2)	0•001
M23	MLM	No root	89•4	(13•4)	-
M23	MLM	+Live root	117•5	(24•5)	0•01
M23	MLM	+Killed root	121•9	(28•5)	0•01

* Number of replicates = 6.

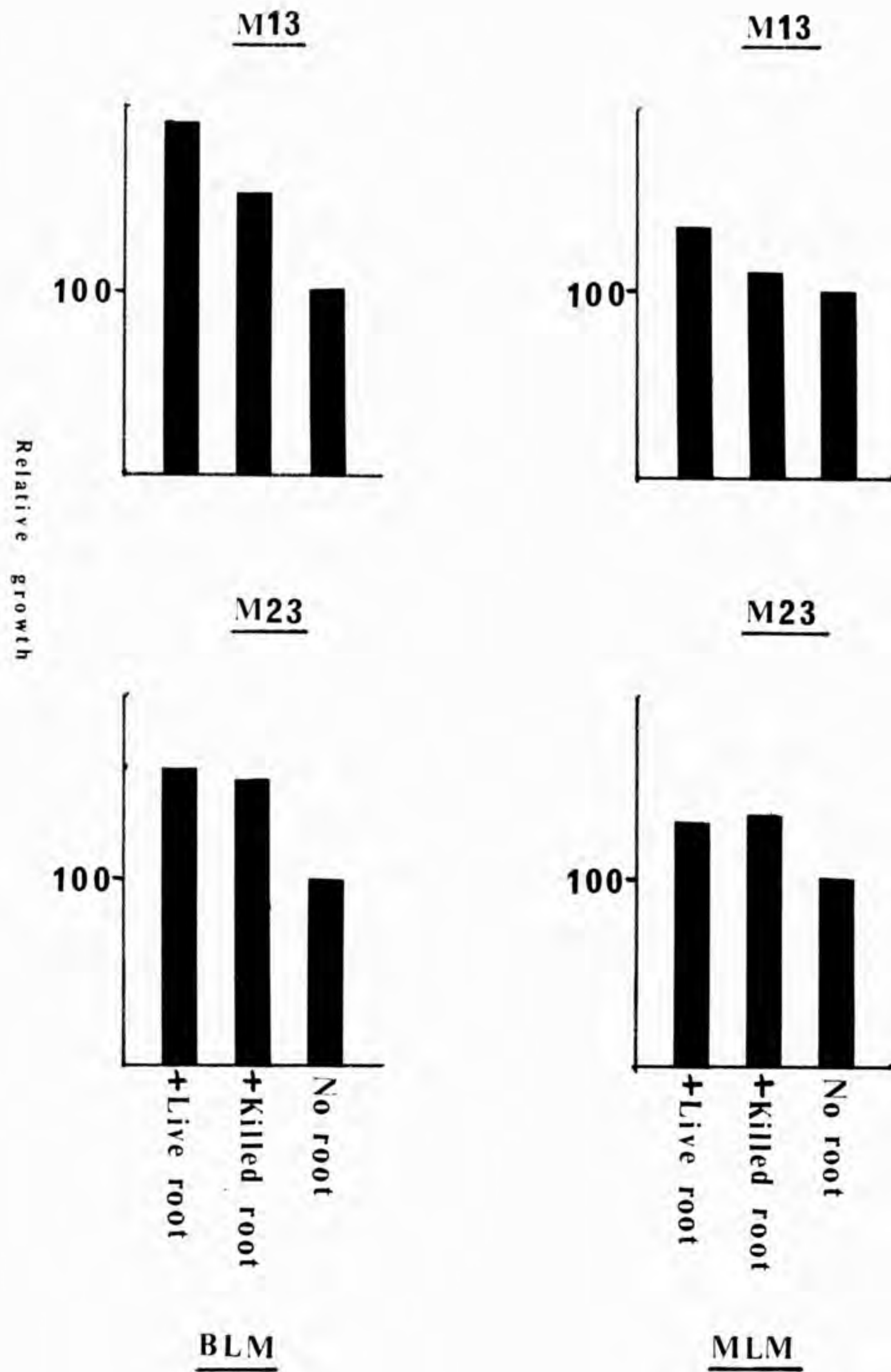


Fig. 29. Growth of M13 and M23 in the presence of excised tomato roots in BLM and MLM for 14 days.

d) <u>Growth of M13 (continuous laboratory culture and culture maintained under oil) in the presence of excised tomato roots in MLM.</u>

Continuous subculturing over a period of time reduced the response of M13 to roots (Table 22).

<u>Table 22.</u>

<u>Growth of M13 (continuous laboratory culture and culture maintained under oil) in the presence of live and killed excised tomato roots in MLM.</u>

Fungus	Treatment	Mean dry weight, mg	Standard error	P
M13 (from continuous laboratory culture)	No root	63•5	(11•5)	-
	+Live root	122•8	(22•2)	0•01
	+Killed root	104•3	(6•5)	0•002
M13 (from culture maintained under oil)	No root	52•3	(9•3)	-
	+Live root	157•8	(20•5)	0•001
	+Killed root	108•2	(21•9)	0•01

e) Growth of M13 in the presence of a cotton "root" and an extracted excised tomato root, both with and without live excised tomato root exudate (micropore filtered).

The growth of M13 in the presence of a cotton "root", with and without live excised tomato root exudate, also an extracted root, with and without live excised tomato root exudate is shown in the following table (Table 23) and Fig. 30.

Table 23.

Growth of M13 in the presence of a cotton "root" and an extracted excised tomato root, both with and without live excised tomato root exudate (micropore filtered).

Type of root	Mean dry weight, mg	Standard error	P
No root (control)	57•6	(9•4)	-
Cotton "root"	86•6	(26•8)	0•05
No root (control)	66•2	(12•2)	-
Cotton "root"+*exudate	108•5	(17•4)	0•01
No root (control)	78•2	(13•6)	-
Extracted root	126•6	(5•6)	0•001
No root (control)	123•7	(25•5)	-
Extracted root+*exudate	215•8	(3•0)	0•001

* Micropore filtered live excised tomato root exudate.

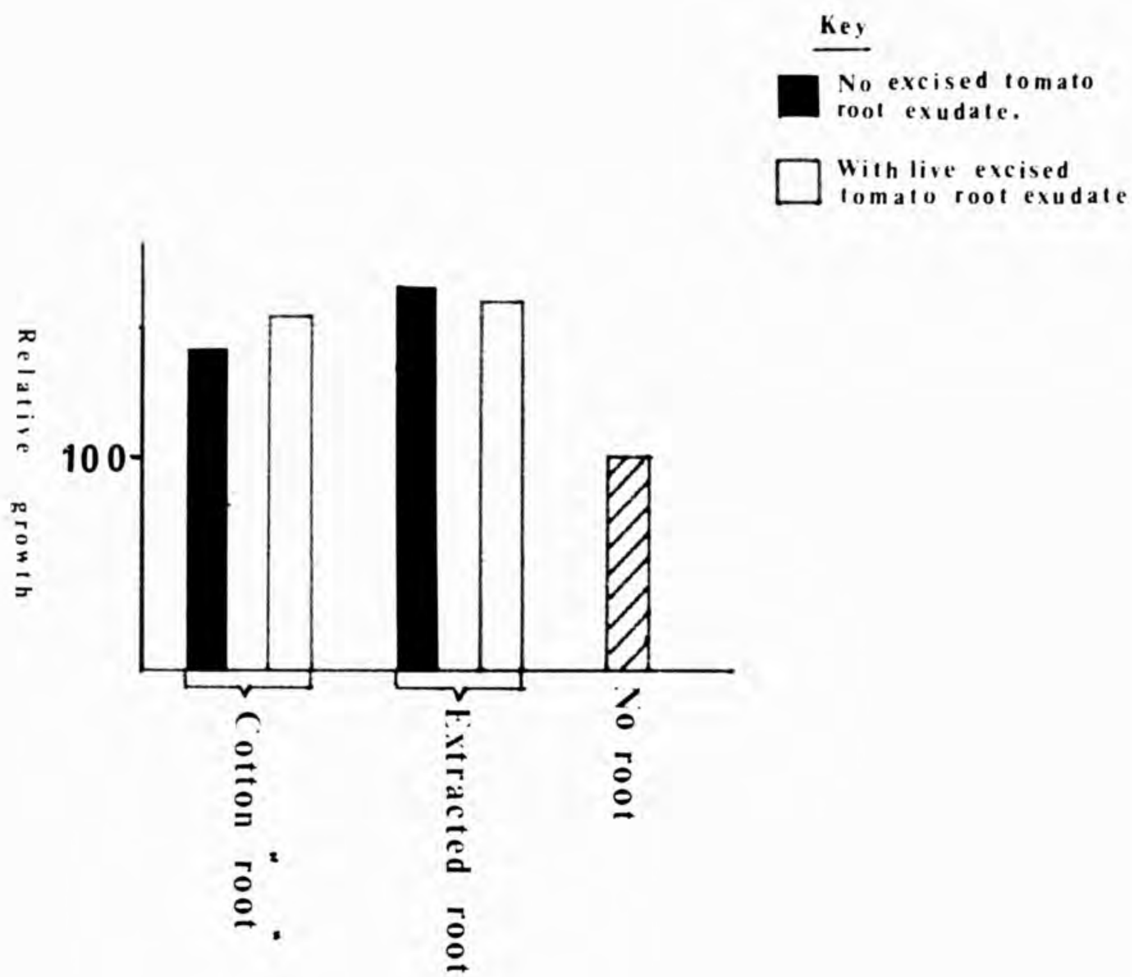


Fig. 30. Growth of M13 in the presence of a cotton "root" and an extracted root, both with and without live excised tomato root exudate (micropore filtered).

These results, with artificial cotton "roots", show that the increase in growth of M13 can be attributed partially, but not wholly to the excised tomato roots acting as a support in the liquid medium. Adding live excised tomato root exudate to the flasks with the cotton "roots" caused an increase in the growth of the fungus, but still the same amount of growth stimulation was not obtained as when a live excised tomato root was present in the medium.

An extracted excised tomato root with and without the presence of live excised tomato root exudate also stimulated the growth of M13, but again not to the same extent as a live excised tomato root, although it does stimulate more than a cotton "root" with live excised tomato root exudate.

f) <u>The effect of enclosing excised tomato roots within dialysis membrane bags on the growth of M13 in MLM.</u>

The effect of separating excised live and killed tomato roots from the fungus by enclosing them within dialysis membrane bags in MLM is shown in Table 24 and Fig. 31.

Table 24.

The effect of enclosing excised tomato roots within dialysis membrane bags on the growth of M13 in MLM.

Treatment	Mean dry weight, mg	Standard error	P
No bag or root (control)	76•6	(17•7)	-
Live root enclosed in bag	10•2	(7•1)	0•001 (inhibition)
Killed root enclosed in bag	21•1	(7•3)	0•002 (inhibition)
No bag or root (control)	57•6	(9•4)	-
Empty bag	27•7	(6•3)	0•01 (inhibition)

Material from live and killed excised tomato roots, which passes through the dialysis membrane inhibited the growth of M13, the effect being greatest with live roots. Inhibition is still obtained with live roots, even after allowance has been made for inhibition due to the dialysis membrane itself.

g) The effect of autoclaving dialysis membrane bags.

The results (see Table 25) indicate that the dialysis membrane does not alter its physical properties when it is autoclaved, for only the ammonium sulphate passed into the external medium, the starch remaining inside the dialysis membrane bags.

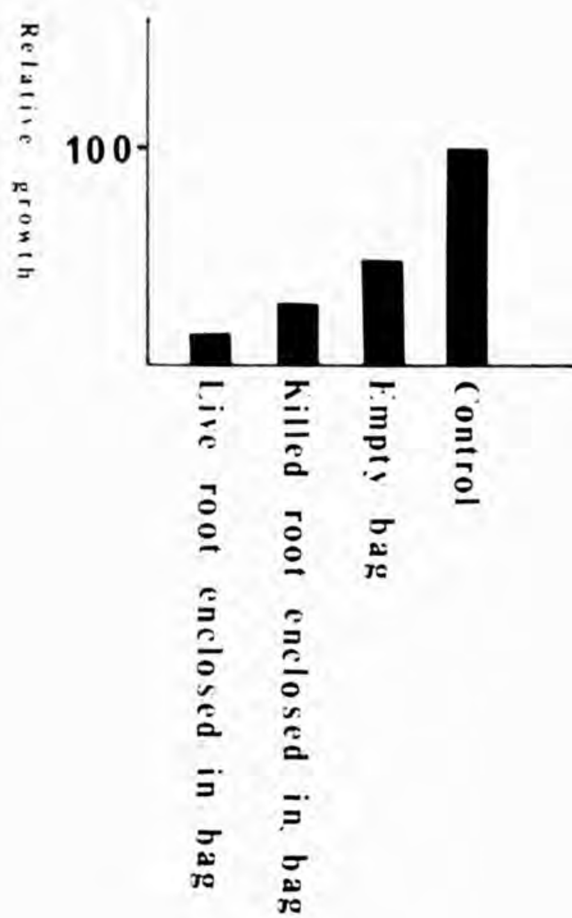


Fig. 31. The effect of enclosing excised tomato roots within dialysis membrane bags on the growth of M13 in MLM.

Table 25.

The effect of autoclaving dialysis membrane bags.

Treatment	Presence or absence in outer medium of:-	
	Ammonium sulphate	Starch
Autoclaved bag	+	-
Non-autoclaved bag	+	-

2. Experiments with excised tomato root exudate, extract and homogenate.

a) Growth of M13 in the presence of excised tomato root exudate, from live and killed roots (collected in distilled water), extract and homogenate in MLM.

The growth of M13 was increased in the presence of micropore filtered and autoclaved excised tomato root exudate (from live and killed roots), extract and homogenate (see Table 26 below and also Fig. 32).

Table 26.

Growth of M13 in the presence of excised tomato root exudate, from live and killed roots (collected in distilled water), extract and homogenate in FLM.

Method of sterilization	Treatment	Mean dry weight, mg	Standard error	P
-	No root preparation (control)	75•7	(12•7)	-
Micropore filtration	Live root exudate	107•9	(6•5)	0•01
Autoclaving	Live root exudate	109•7	(23•9)	0•02
Micropore filtration	Killed root exudate	106•8	(11•8)	0•02
Autoclaving	Killed root exudate	165•4	(12•0)	0•001
-	No root preparation (control)	117•9	(2•7)	-
Micropore filtration	Extract	224•5	(43•3)	0•01
Autoclaving	Extract	195•7	(44•8)	0•02
-	No root preparation (control)	70•2	(13•9)	-
Micropore filtration	Homogenate	136•9	(18•3)	0•002
Autoclaving	Homogenate	135•5	(24•8)	0•01

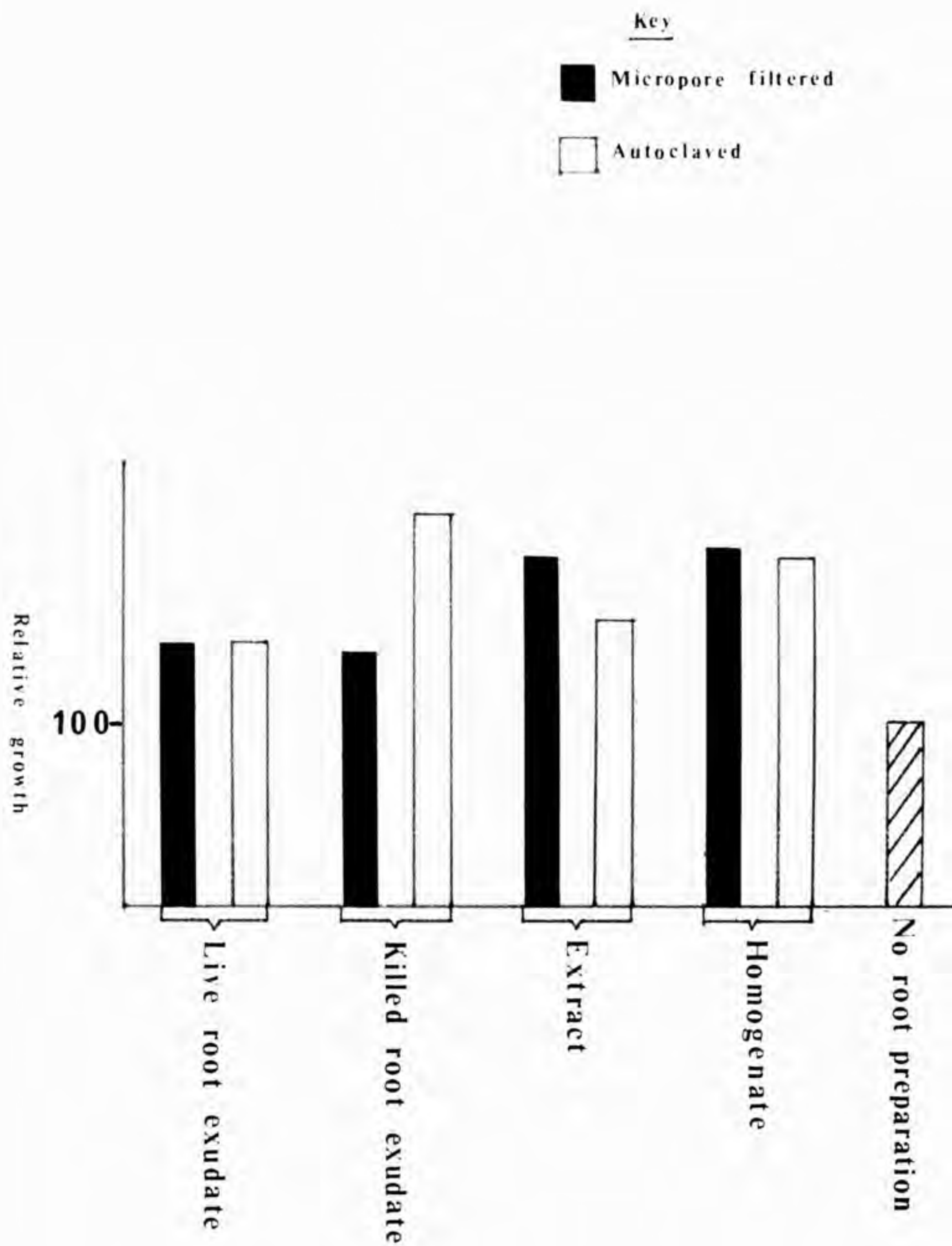


Fig. 32. Growth of M13 in the presence of excised tomato root exudate from live and killed roots (collected in distilled water), extract and homogenate in MLM.

The most significant growth stimulation, after sterilizing by micropore filtration, was produced with excised tomato root homogenate. Slightly less growth stimulation occurred in the presence of excised tomato root extract and the least with excised tomato root exudates (from live and killed roots). Autoclaving the root preparations slightly reduced the ability of the live excised tomato root exudate, extract and homogenate to stimulate, but greatly increased the effect of the killed excised tomato root exudate.

b) Growth of M13 at two different levels of micropore filtered excised tomato root exudate, from live and killed roots (collected in distilled water), extract and homogenate in MLM.

Growth of M13 in the presence of two different levels of excised tomato root exudate (from live and killed roots), extract and homogenate is given in Table 27 and Fig. 33.

Table 27.

Growth of M13 at two different levels of micropore filtered excised tomato root exudate from live and killed roots (collected in distilled water), extract and homogenate in MLM.

Quantity of root preparation (dry weight of excised tomato root)	Treatment	Mean dry weight, mg	Standard error	P
None	No root preparation (control)	75•2	(19•4)	-
6•2mg	Live root exudate	90•3	(14•6)	n.s.
3•1mg	Live root exudate	71•1	(8•4)	n.s.
6•2mg	Killed root exudate	106•8	(10•0)	0•02
3•1mg	Killed root exudate	97•1	(17•9)	n.s.
None	No root preparation (control)	71•7	(4•9)	-
6•2mg	Extract	128•4	(4•8)	0•001
3•1mg	Extract	101•2	(19•5)	0•02
6•2mg	Homogenate	135•7	(12•4)	0•001
3•1mg	Homogenate	116•7	(6•7)	0•001

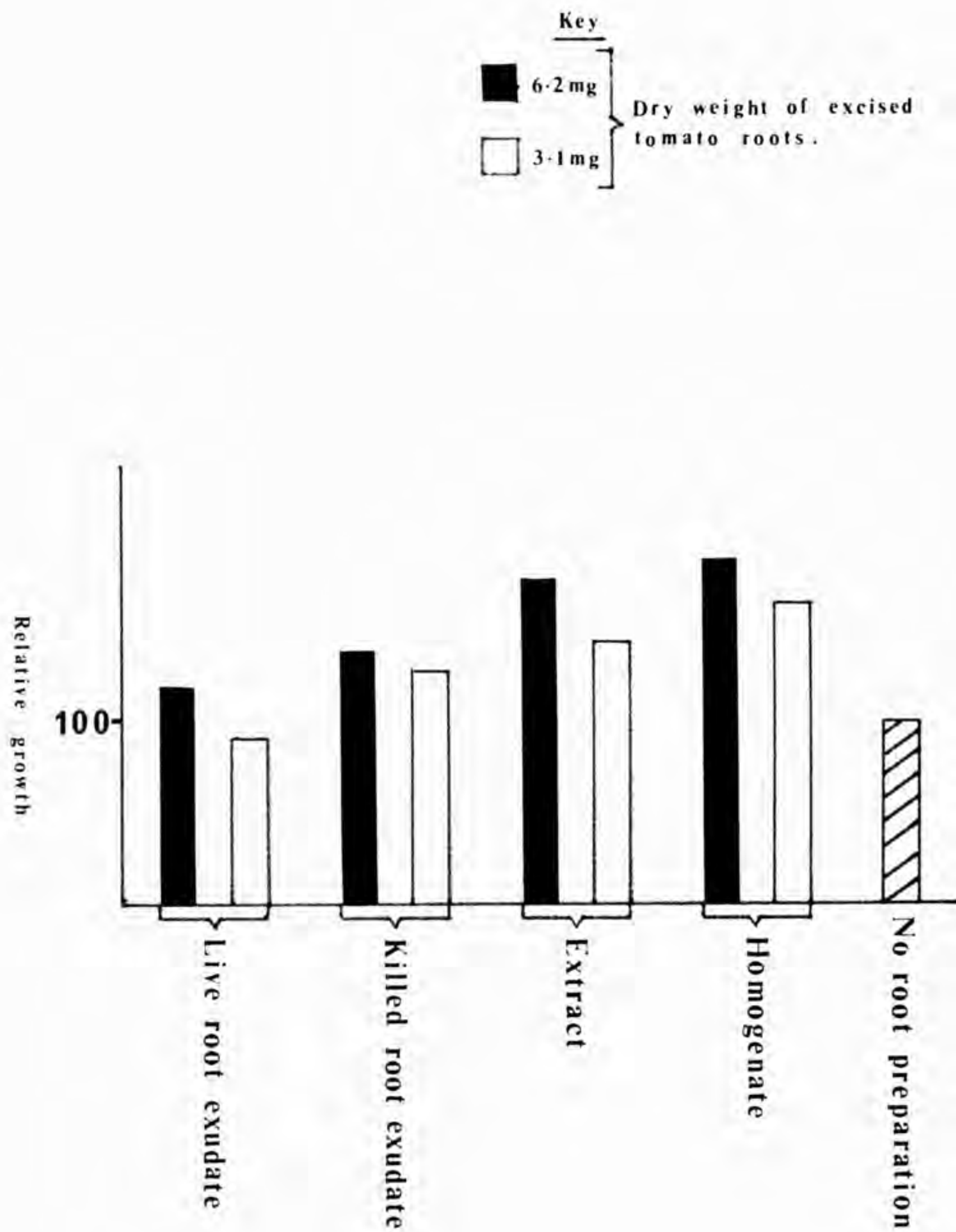


Fig. 33. Growth of M13 at two different levels of micropore filtered excised tomato root exudate from live and killed roots (collected in distilled water), extract and homogenate in MLM.

These results indicate that the amount of stimulation obtained depends on the quantity of root preparation added, 3·1mg dry weight of excised tomato root giving less stimulation than 6·2mg, except live excised tomato root exudate, where neither 3·1mg or 6·2mg dry weight of excised tomato root significantly stimulated the growth of M13.

c) Growth of M13 in the presence of excised tomato root exudate collected daily (in distilled water) in MLM.

The growth of M13 in the presence of excised tomato root exudate collected daily and then pooled, also collected daily and added daily to the growth flasks are shown in Table 28 and Fig. 34.

Table 28.

Growth of M13 in the presence of excised tomato root exudate collected daily (in distilled water) in MLM.

Type of exudate	Treatment	Method of sterilization	Mean dry weight, mg	Standard error	P
-	No exudate (control)	-	73•2	(14•2)	-
From live roots	Pooled	Micropore filtration	70•6	(18•6)	n.s.
From live roots	Pooled	Autoclaving	118•6	(6•2)	0•01
From killed roots	Pooled	Micropore filtration	76•5	(12•5)	n.s.
From killed roots	Pooled	Autoclaving	96•9	(14•2)	0•05
-	No exudate (control)	-	79•3	(3•6)	-
From live roots	Added daily	Micropore filtration	57•6	(3•6)	0•001*
-	No exudate (control)	-	55•4	(1•9)	-
From live roots	Added daily	Autoclaving	70•1	(0•2)	0•001
From killed roots	Added daily	Micropore filtration	66•9	(8•4)	0•02
From killed roots	Added daily	Autoclaving	68•7	(6•0)	0•01

* Inhibition.

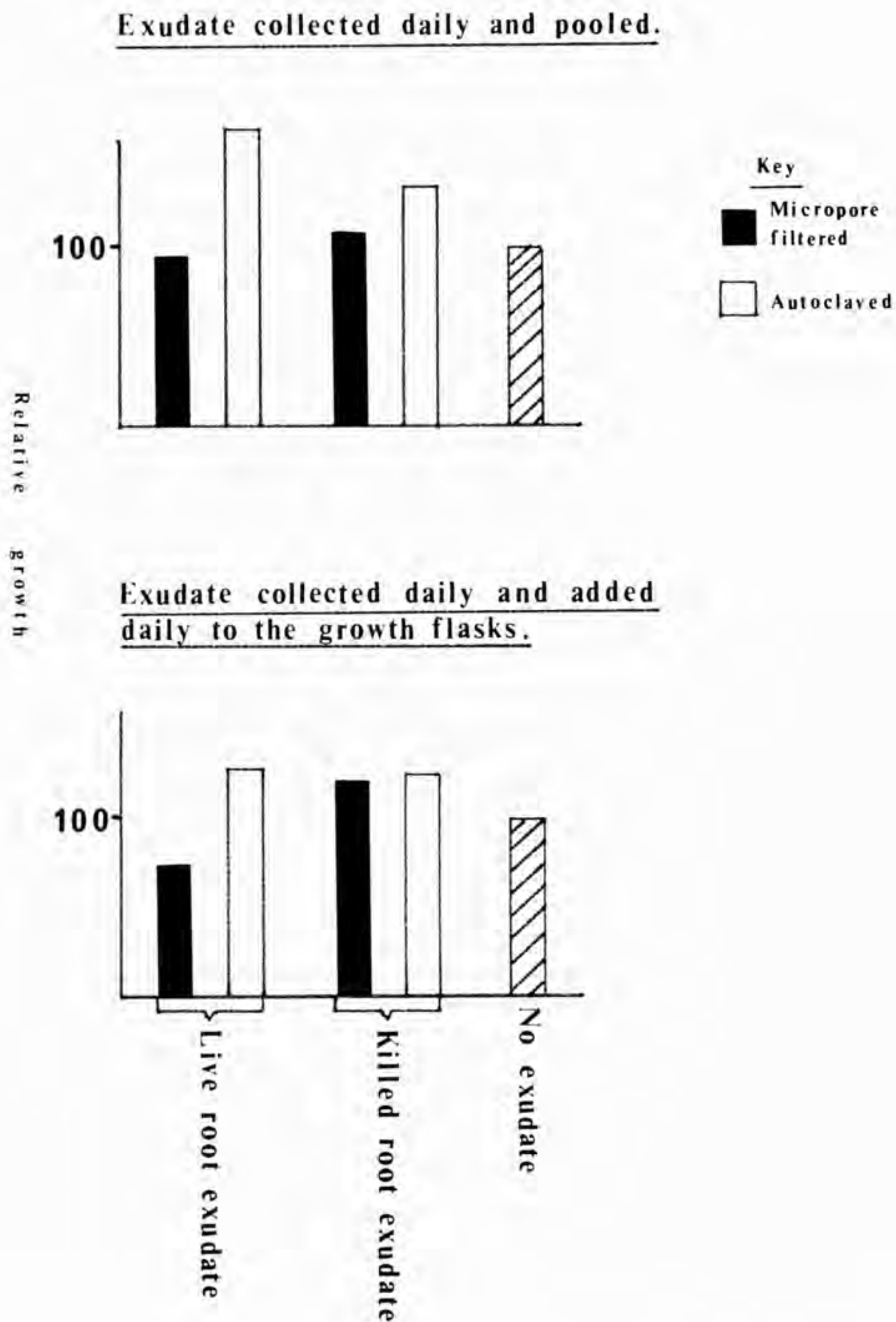


Fig. 34. Growth of M13 in the presence of excised tomato root exudate collected daily (in distilled water) in MLM.

The killed and live excised tomato root exudates collected daily and pooled had no significant effect on the growth of M13, but when these root preparations were autoclaved stimulation of growth occurred. Live exudate collected daily and added daily inhibited growth, but the killed exudate caused growth stimulation. Autoclaving both these root preparations stimulated the growth of M13.

d) <u>Growth of M13 in the presence of dialysate and retentate of tomato root exudate (from live excised tomato roots, collected in distilled water), extract and homogenate in MLM.</u>

The effect of dialysing live excised tomato root exudate, extract and homogenate against water on the growth of M13 is shown in Table 29 and Fig. 35.

Table 29.

Growth of M13 in the presence of dialysate and retentate of tomato root exudate (from live excised tomato roots, collected in distilled water), extract and homogenate in MLM.

Method of sterilization	Treatment	Mean dry weight, mg	Standard error	P
-	No root preparation (control)	75•7	(12•7)	-
Micropore filtration	Dialysate of live root exudate	94•0	(11•5)	0•05
Autoclaving	Dialysate of live root exudate	91•0	(5•5)	0•05
Micropore filtration	Retentate of live root exudate	95•2	(5•7)	0•05
Autoclaving	Retentate of live root exudate	89•9	(11•3)	n.s.
-	No root preparation (control)	117•9	(2•7)	-
Micropore filtration	Dialysate of extract	167•5	(13•2)	0•001
Autoclaving	Dialysate of extract	197•7	(21•8)	0•001
Micropore filtration	Retentate of extract	169•5	(36•3)	0•05
Autoclaving	Retentate of extract	200•2	(67•6)	0•05
-	No root preparation (control)	70•2	(13•9)	-
Micropore filtration	Dialysate of homogenate	124•5	(10•0)	0•002
Autoclaving	Dialysate of homogenate	105•9	(10•5)	0•01
Micropore filtration	Retentate of homogenate	118•9	(11•1)	0•01
Autoclaving	Retentate of homogenate	122•8	(8•9)	0•002

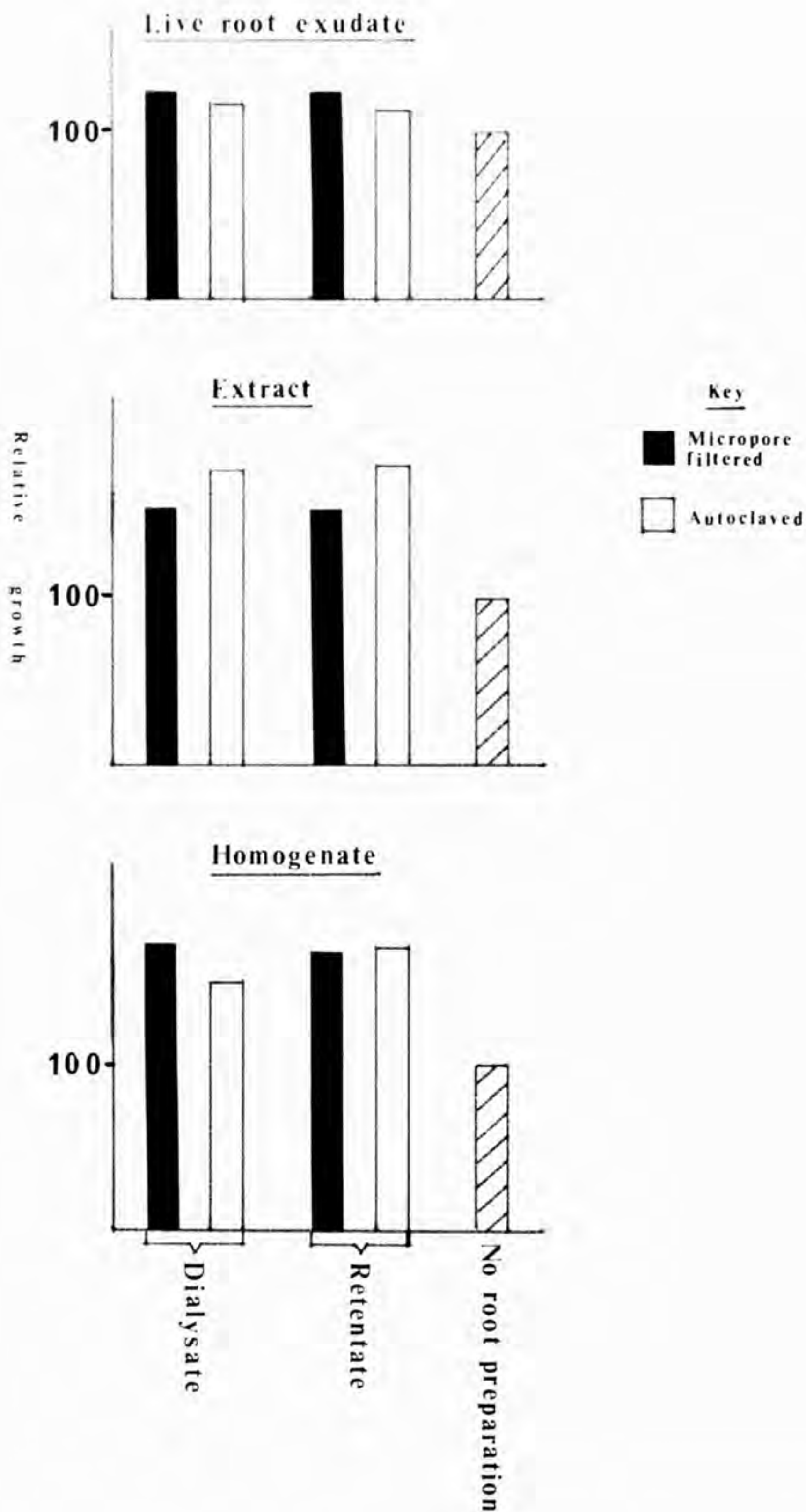


Fig. 35. Growth of M13 in the presence of dialysate and retentate of tomato root exudate (from live excised tomato roots, collected in distilled water), extract and homogenate in MLM.

These results suggest that the stimulatory factor(s) consists of two fractions, one dialysable and one not, each of which stimulated the fungus to approximately the same extent.

e) Growth of M13 in MLM, in the presence of dialysate and retentate collected in distilled water from live exuding excised tomato roots enclosed within dialysis membrane bags.

This experiment was repeated three times, but conflicting results were obtained as shown below (see Table 30 and Fig. 36).

Table 30.

Growth of M13 in FLM, in the presence of dialysate and retentate collected in distilled water from live exuding excised tomato roots enclosed within dialysis membrane bags.

Method of sterilization of exudate	Treatment	Mean dry weight, mg	Standard error	P	Experiment number
-	No exudate (control)	89·6	(12·2)	-	1
Micropore filtration	Dialysate	48·1	(63·0)	n.s.	1
Autoclaving	Dialysate	48·0	(11·4)	0·002	1
Micropore filtration	Retentate	93·4	(4·4)	n.s.	1
Autoclaving	Retentate	81·4	(8·5)	n.s.	1
-	No exudate (control)	83·4	(12·2)	-	2
Micropore filtration	Dialysate	118·4	(9·3)	0·01	2
Autoclaving	Dialysate	115·3	(6·1)	0·01	2
Micropore filtration	Retentate	104·9	(3·7)	0·01	2
Autoclaving	Retentate	106·9	(6·6)	0·01	2
-	No exudate (control)	138·7	(7·1)	-	3
Micropore filtration	Dialysate	144·8	(24·1)	n.s.	3
Autoclaving	Dialysate	173·5	(11·3)	0·002	3
Micropore filtration	Retentate	181·1	(27·3)	0·02	3
Autoclaving	Retentate	134·1	(20·4)	n.s.	3

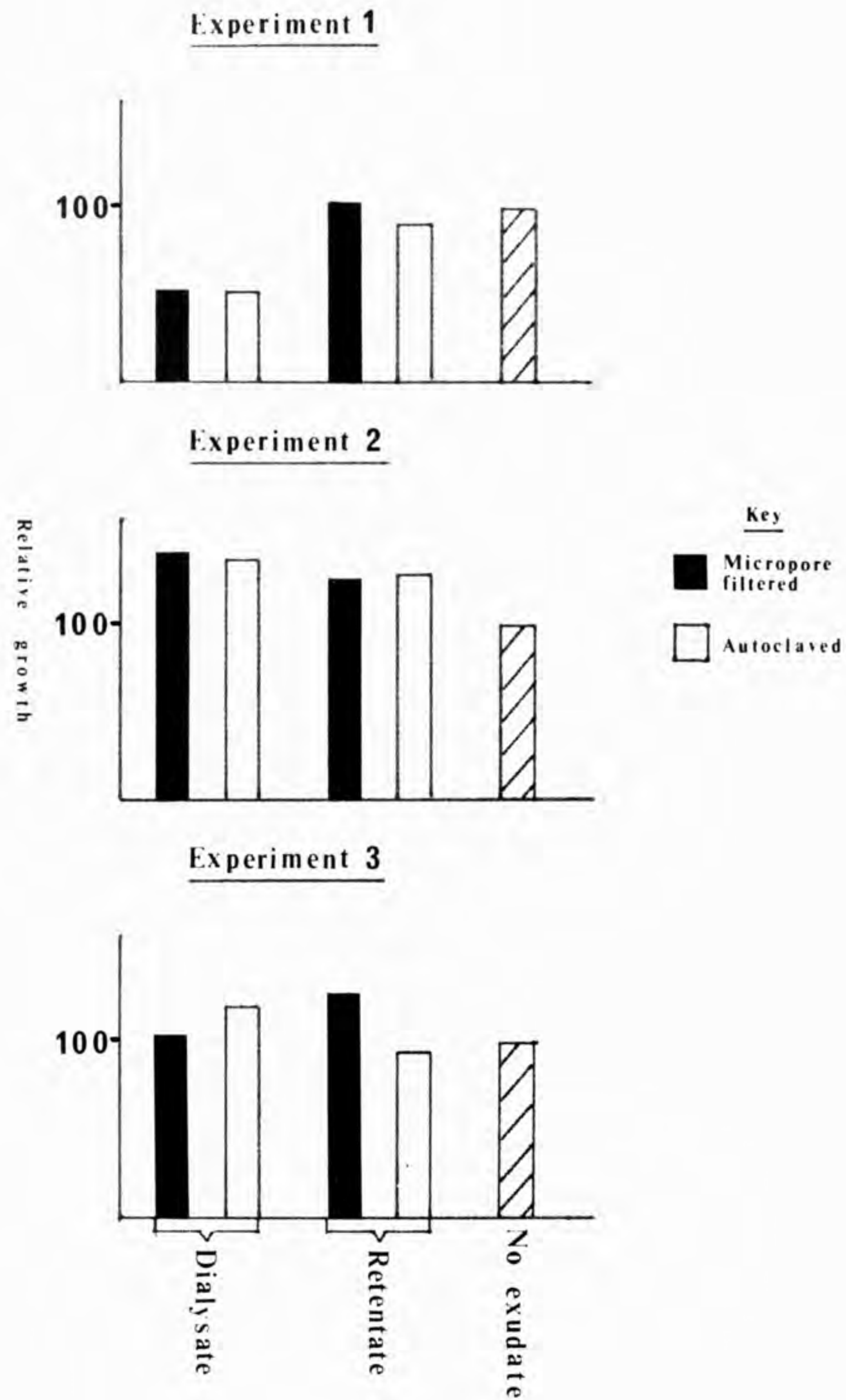


Fig. 36. Growth of M13 in MLM, in the presence of dialysate and retentate collected in distilled water from live exuding excised tomato roots enclosed within dialysis membrane bags.

In the first experiment the micropore filtered dialysate and retentate and the autoclaved retentate had no significant effect on the growth of M13. The autoclaved dialysate stimulated. In the second experiment growth stimulation occurred with all samples, but in the third experiment stimulation of growth was only obtained with the autoclaved retentate and the micropore filtered dialysate. The reason for these conflicting results is unknown.

f) Growth of M13 in MLM, in the presence of live excised tomato root exudate (collected in MLM).

The results of an experiment in which live excised tomato root exudate was collected in MLM and tested on M13 are given in Table 31 and Fig. 37.

Table 31.

Growth of M13 in MLM, in the presence of live excised tomato root exudate (collected in MLM).

Treatment	Mean dry weight, mg	Standard error	P
No exudate (control)	76•8	(13•3)	-
Live root exudate	256•1	(81•5)	0•01
Live root exudate (autoclaved)	296•2	(31•1)	0•001

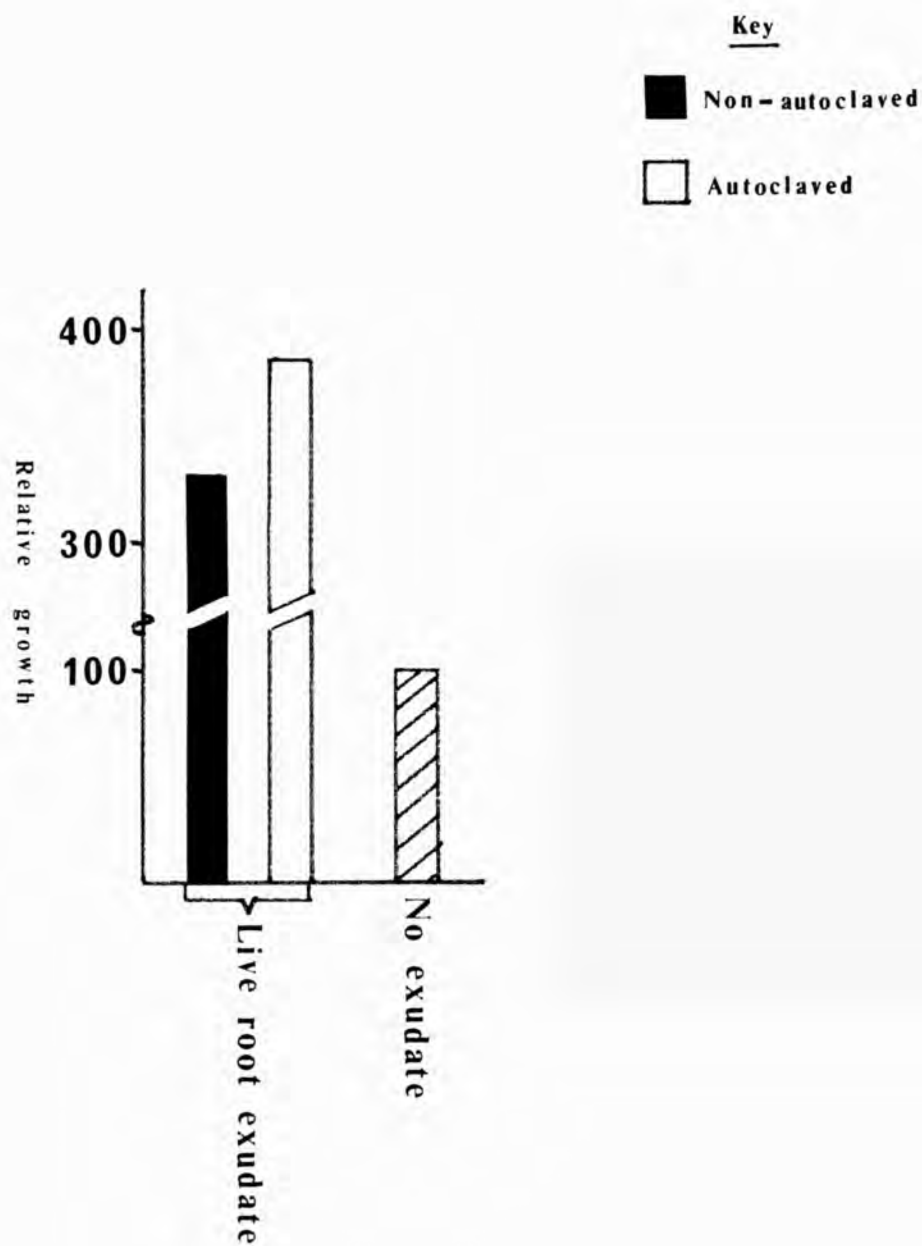


Fig. 37. Growth of M13 in MLM, in the presence of live excised tomato root exudate (collected in MLM).

Exudate of live excised tomato roots collected in MLM stimulated the growth of M13, the level of stimulation being much greater than that with exudate collected in water, but very similar to that obtained in the presence of excised live tomato roots after 14 days growth (see Fig. 27).

g) Growth of M13 in MLM, in the presence of dialysate and retentate collected in MLM from live exuding excised tomato roots enclosed within dialysis membrane bags.

The growth of M13 in the presence of dialysate and retentate collected after the dialysis of live exuding tomato roots (against MLM) is shown in Table 32 and Fig. 38.

Table 32.

Growth of M13 in MLM, in the presence of dialysate and retentate collected in MLM from live exuding excised tomato roots enclosed within dialysis membrane bags.

Method of sterilization	Treatment	Mean dry weight, mg	Standard error	P
-	No exudate (control)	83•4	(12•2)	-
Micropore filtration	Dialysate	63•3	(16•0)	0•05 (inhibition)
Autoclaving	Dialysate	28•5	(22•7)	0•01 (inhibition)
-	No exudate (control)	51•4	(3•0)	-
Micropore filtration	Retentate	69•9	(1•5)	0•001
Autoclaving	Retentate	0•0	(0•0)	Complete inhibition

Dialysate inhibited the growth of M13, whereas retentate stimulated its growth. Autoclaving increased the inhibition produced by the dialysate and caused complete inhibition in the presence of the retentate.

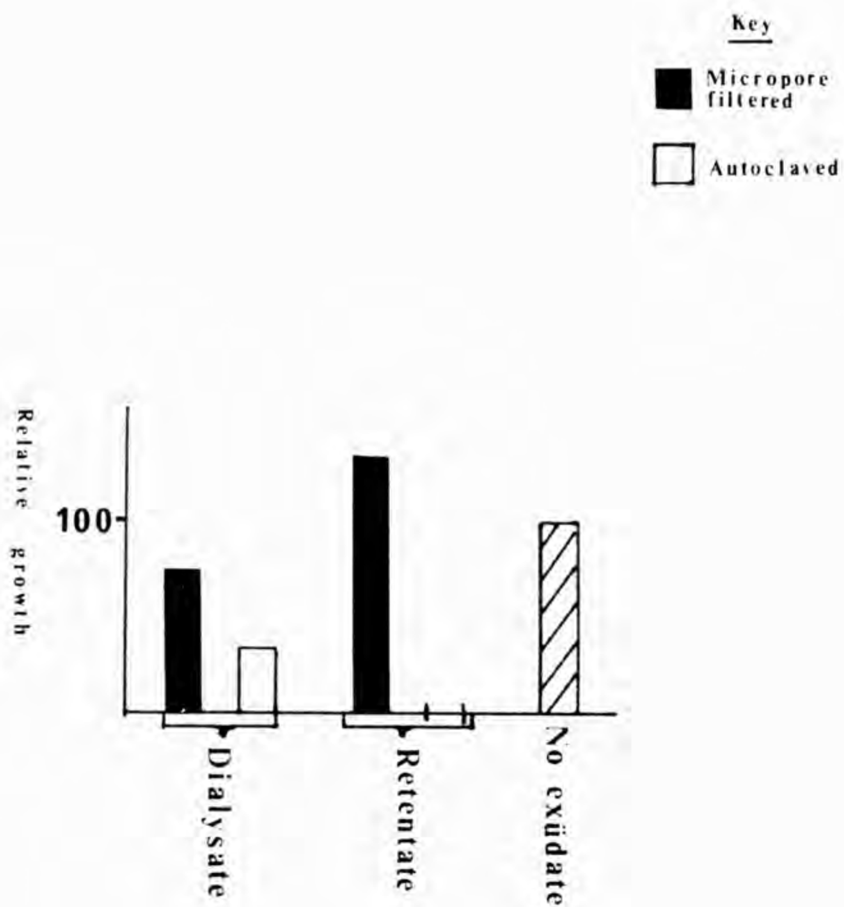


Fig. 38. Growth of M13 in MLM, in the presence of dialysate and retentate collected in MLM from live exuding excised tomato roots enclosed within dialysis membrane bags.

3. <u>The effect of excised tomato root exudate from live and killed roots (collected in distilled water), extract and homogenate on the growth of *Boletus variegatus* 1 in MLM.</u>

The growth of *Boletus variegatus* 1 in the presence of live excised tomato root exudate, extract and homogenate is shown below (Table 33 and Fig. 39).

Table 33.

The effect of excised tomato root exudate from live and killed roots (collected in distilled water), extract and homogenate on the growth of Boletus variegatus 1 in MLM.

Method of sterilization	Treatment	Mean dry weight, mg	Standard error	P
-	No root preparation (control)	35•2	(2•3)	-
Micropore filtration	Live root exudate	23•3	(4•8)	0•01*
Autoclaving	Live root exudate	26•5	(6•1)	0•02*
Micropore filtration	Killed root exudate	12•9	(2•3)	0•001*
Autoclaving	Killed root exudate	13•2	(5•2)	0•001*
-	No root preparation (control)	14•3	(8•3)	-
Micropore filtration	Extract	15•0	(3•9)	n.s.
Autoclaving	Extract	6•3	(0•6)	n.s.
-	No root preparation (control)	3•0	(0•9)	-
Micropore filtration	Homogenate	15•5	(2•4)	0•001
-	No root preparation (control)	13•2	(5•1)	-
Autoclaving	Homogenate	8•9	(2•7)	n.s.

* Inhibition

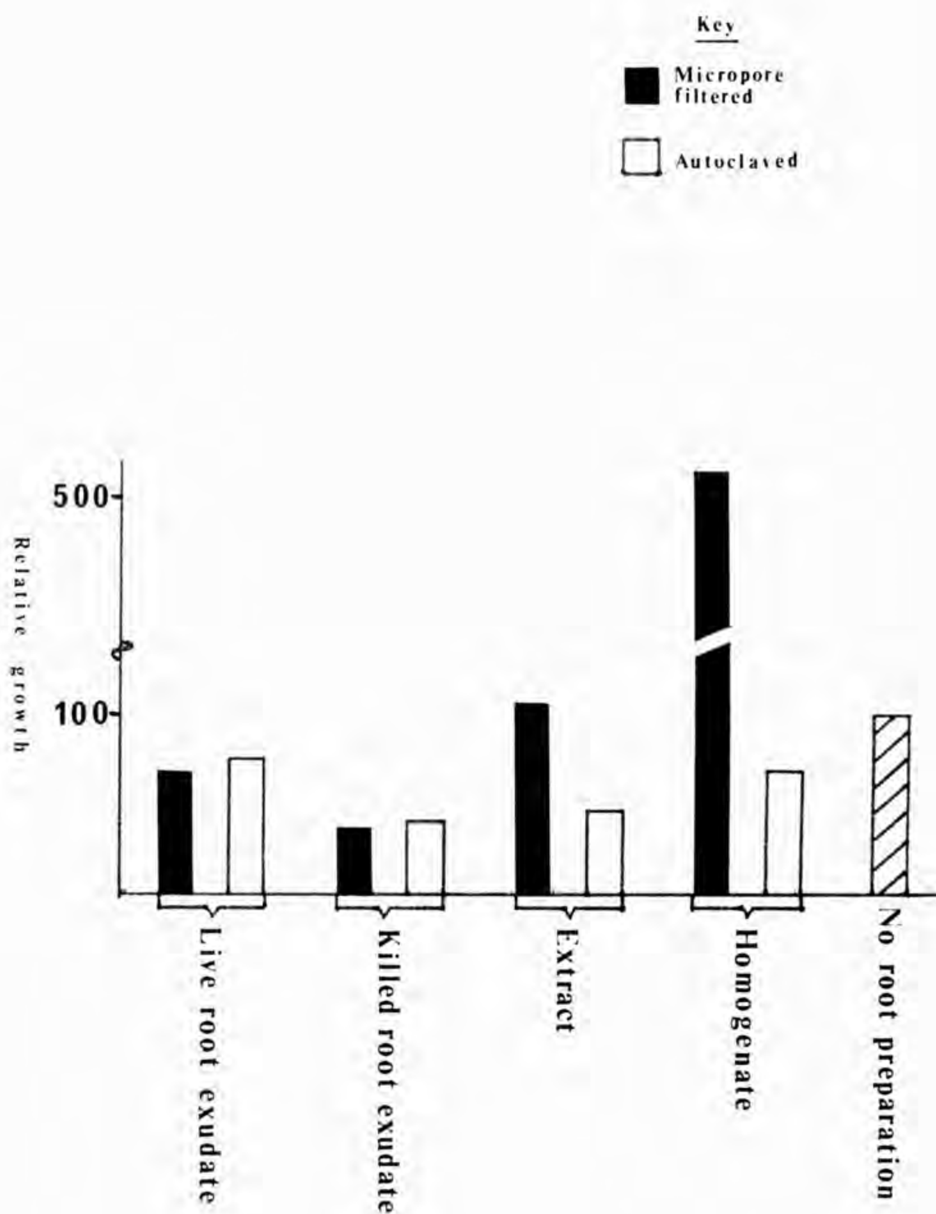


Fig. 39. The effect of excised tomato root exudate, from live and killed roots (collected in distilled water), extract and homogenate on the growth of Boletus variegatus 1, in MLM.

The only excised tomato root preparation which significantly stimulated the growth of Boletus variegatus 1 was the micropore filtered homogenate ($P = 0 \cdot 001$), the rest of the root preparations had no significant growth stimulatory effect. The killed and live excised tomato root preparations all caused growth inhibition.

4. Chromatographic bioassay of excised tomato root preparations.

Definite Rf fractions stimulated the growth of M13, after the fractionation of live excised tomato root exudate, extract and homogenate by paper chromatography, as shown in Figs. 40 to 46 (also see Appendix D).

From Figs. 40, 42, 43 and 45 a definite and consistent pattern of stimulatory Rf fractions were produced by all the root preparations fractionated in BuA and sterilized by UV. Peak Rf fractions were 3, 4 and 6 for exudate, 1, 2, 3, 4, 8 and 10 for extract and 3 and 10 for homogenate. The dialysable Rf fractions (from live exuding excised tomato roots, enclosed within dialysis membrane bags and collected in distilled water) also peaked at 1, 2, 3 and 4 and to a lesser extent at 5, 6, 7 and 8. Similar growth stimulatory Rf fractions were obtained after these fractions had been sterilized by autoclaving (Figs. 40, 42, 43 and 45), except Rf fractions 1, 8 and 10 of the fractionated excised tomato root extract had no effect on the growth of M13 after autoclaving, whereas Rf fraction 5 became growth stimulatory. None of the autoclaved Rf fractions of the dialysate (from live exuding excised tomato roots, enclosed within dialysis membrane bags and collected in distilled water) produced significant growth promotion.

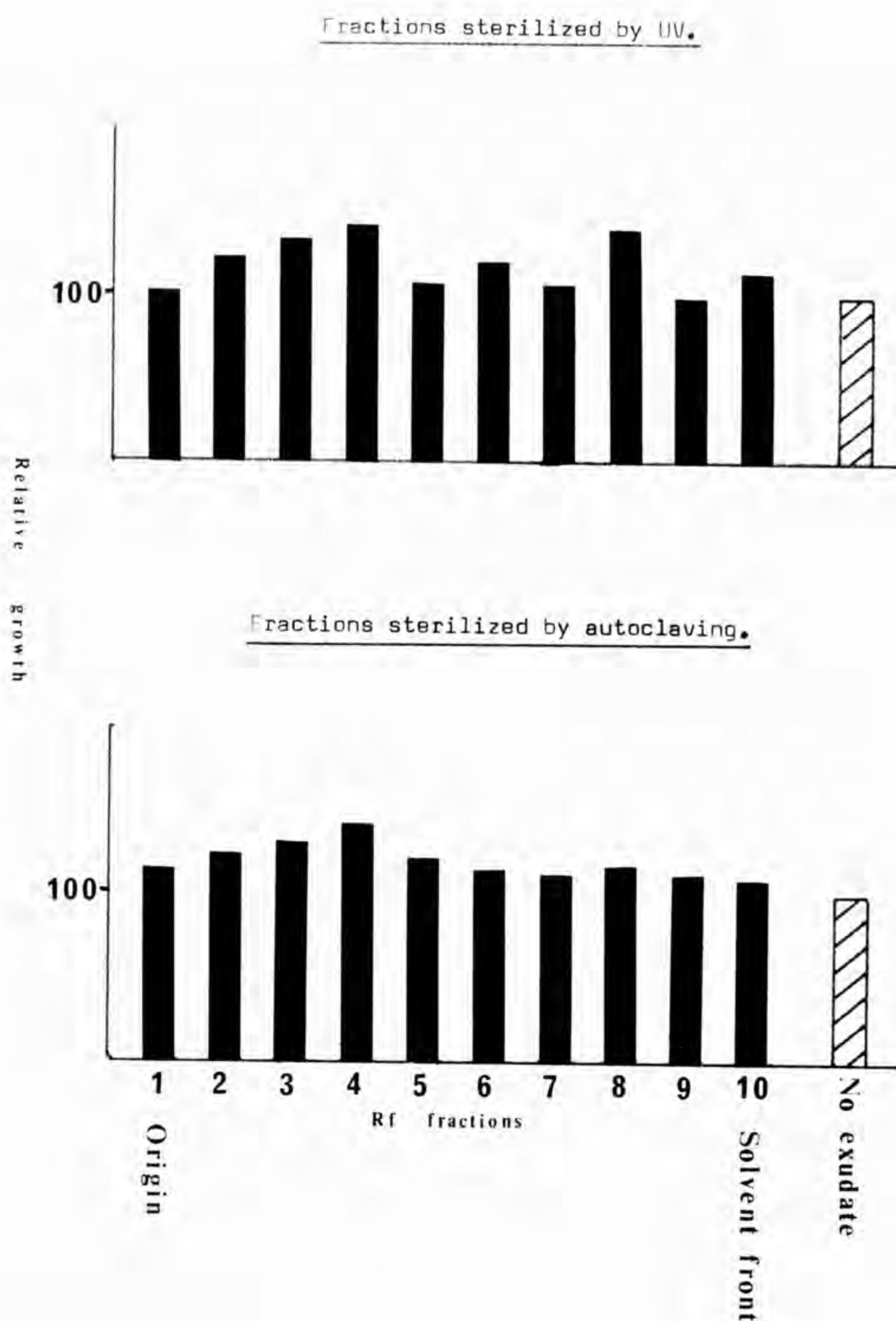


Fig. 40. Growth of M13 in MLM, with different Rf fractions, after the fractionation of live excised tomato root exudate in BuA by paper chromatography.

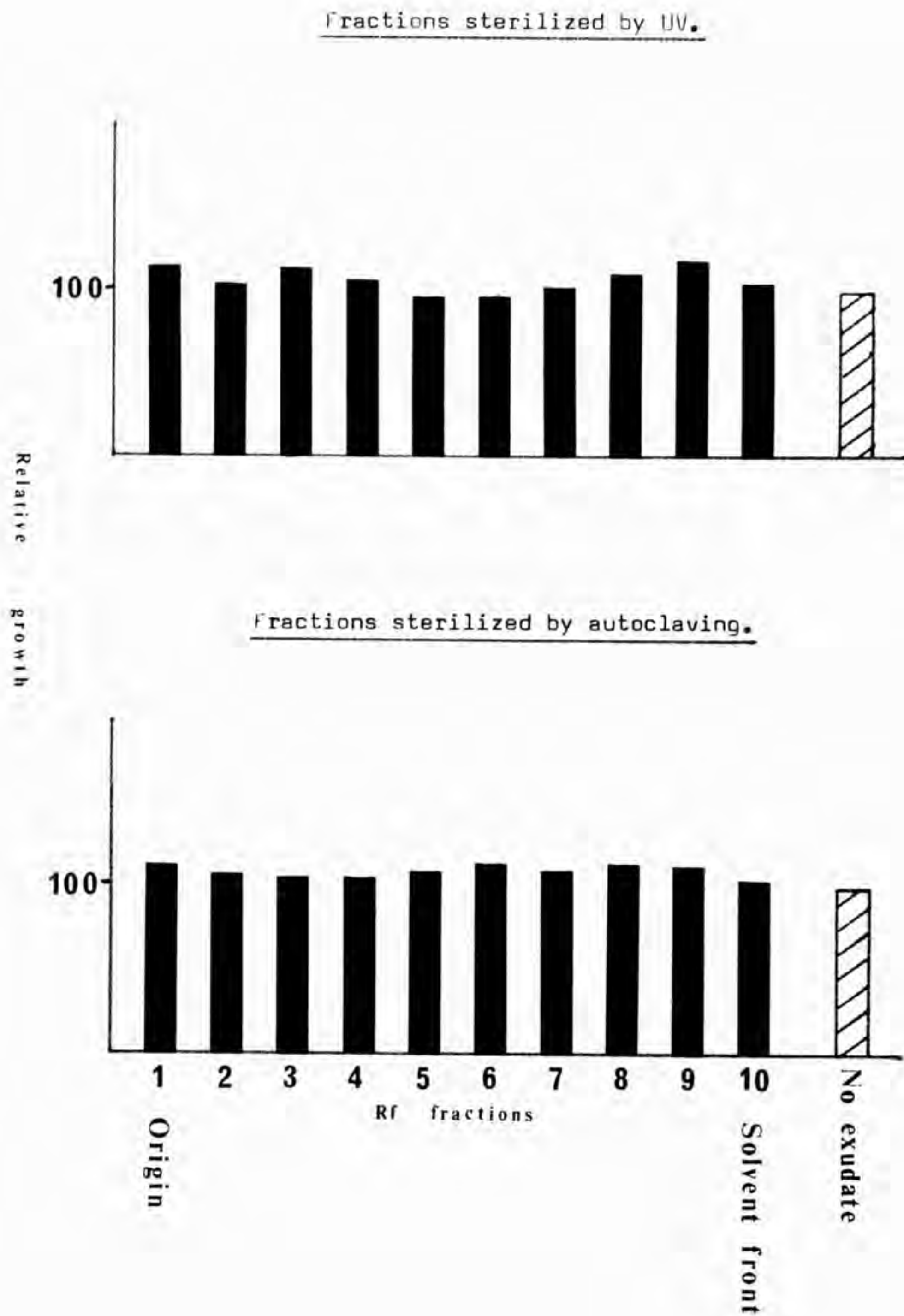


Fig. 41. Growth of M13, in MLM, with different Rf fractions, after the fractionation of live excised tomato root exudate in BuP, by paper chromatography.

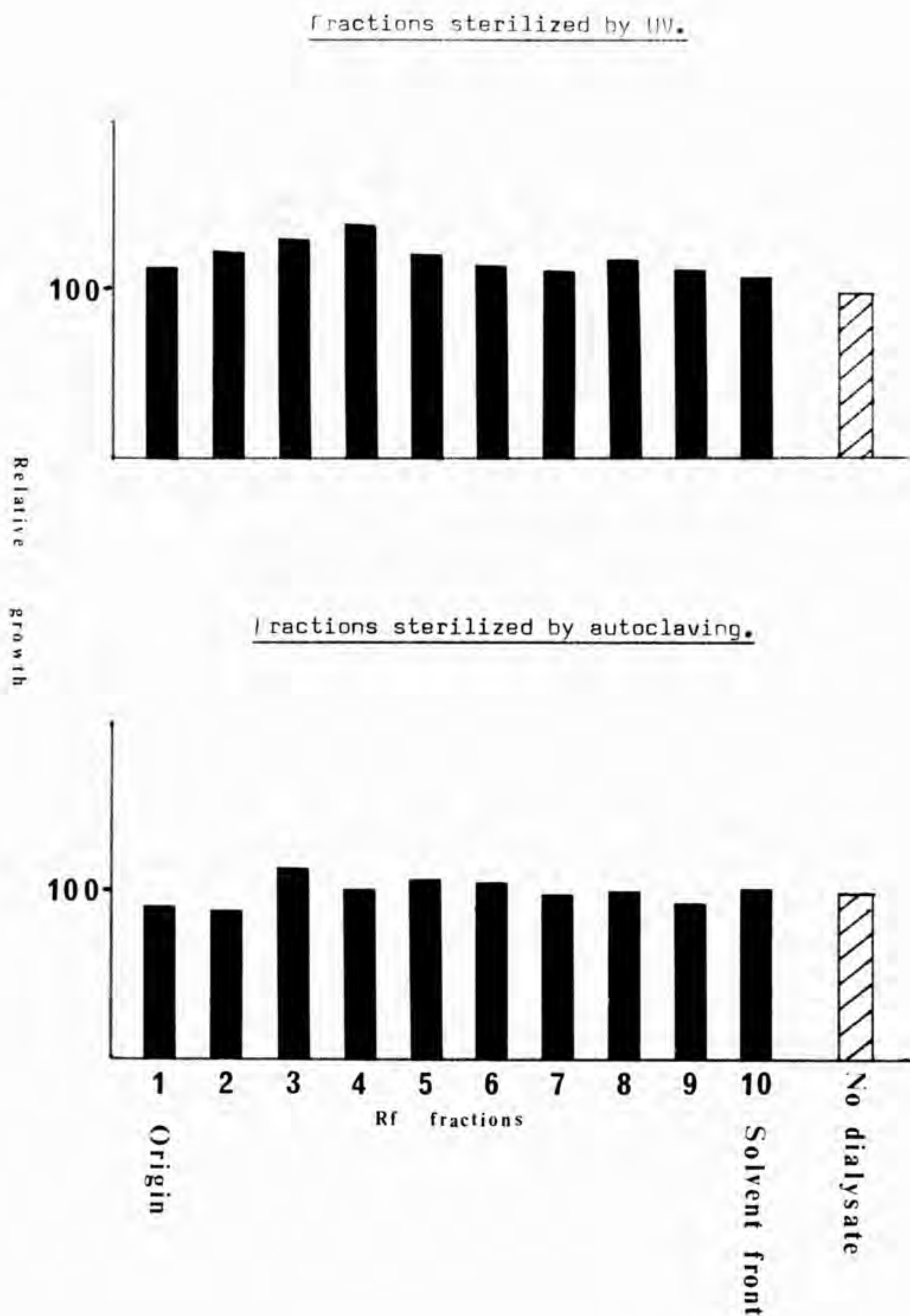


Fig. 42. Growth of M13, in MLM, with different Rf fractions, after the fractionation of live excised tomato root dialysate (collected in distilled water from exuding roots enclosed within dialysis membrane bags) in BuA, by paper chromatography.

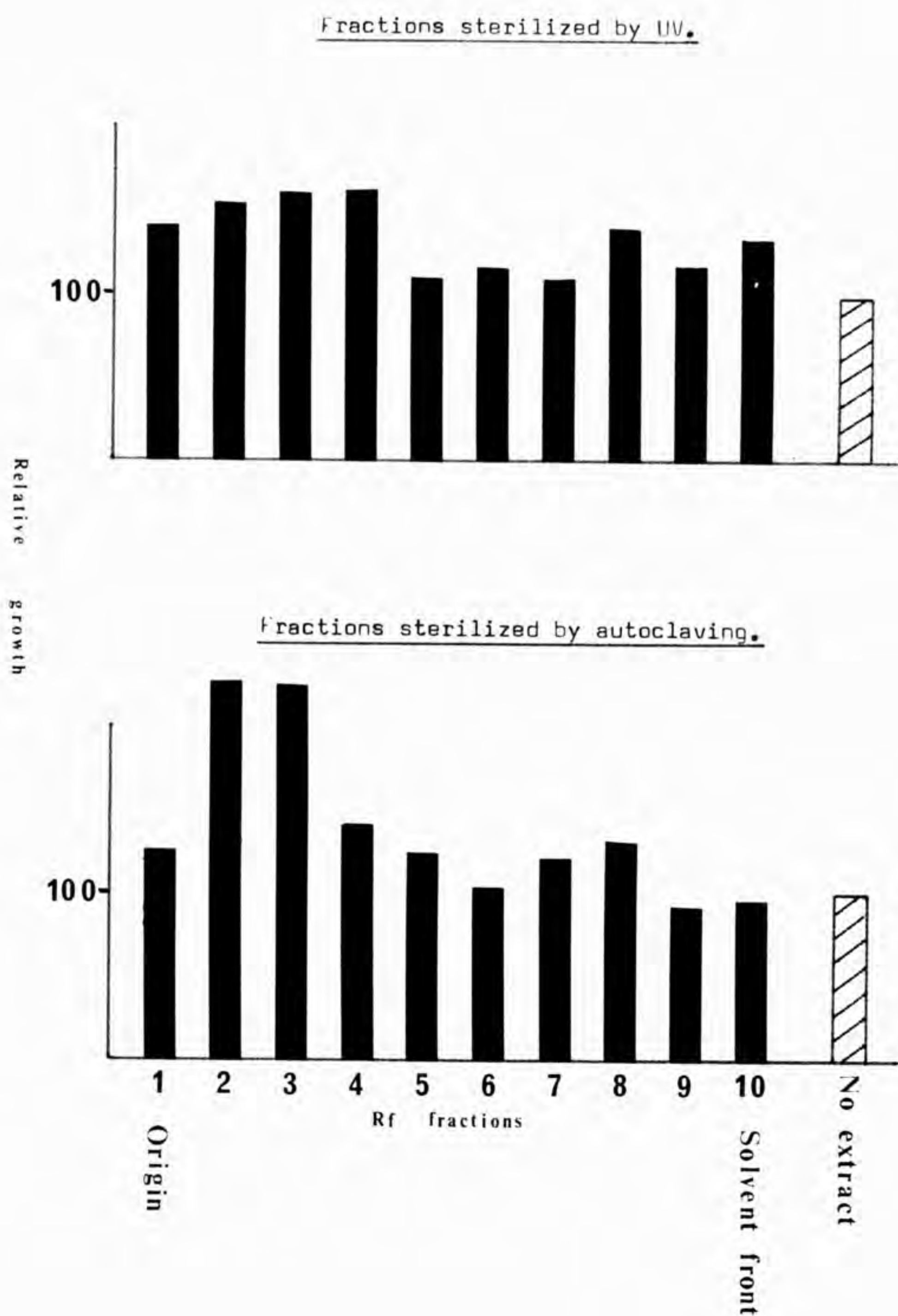


Fig. 43. Growth of M13, in MLM, with different Rf fractions, after the fractionation of excised tomato root extract in BuA, by paper chromatography.

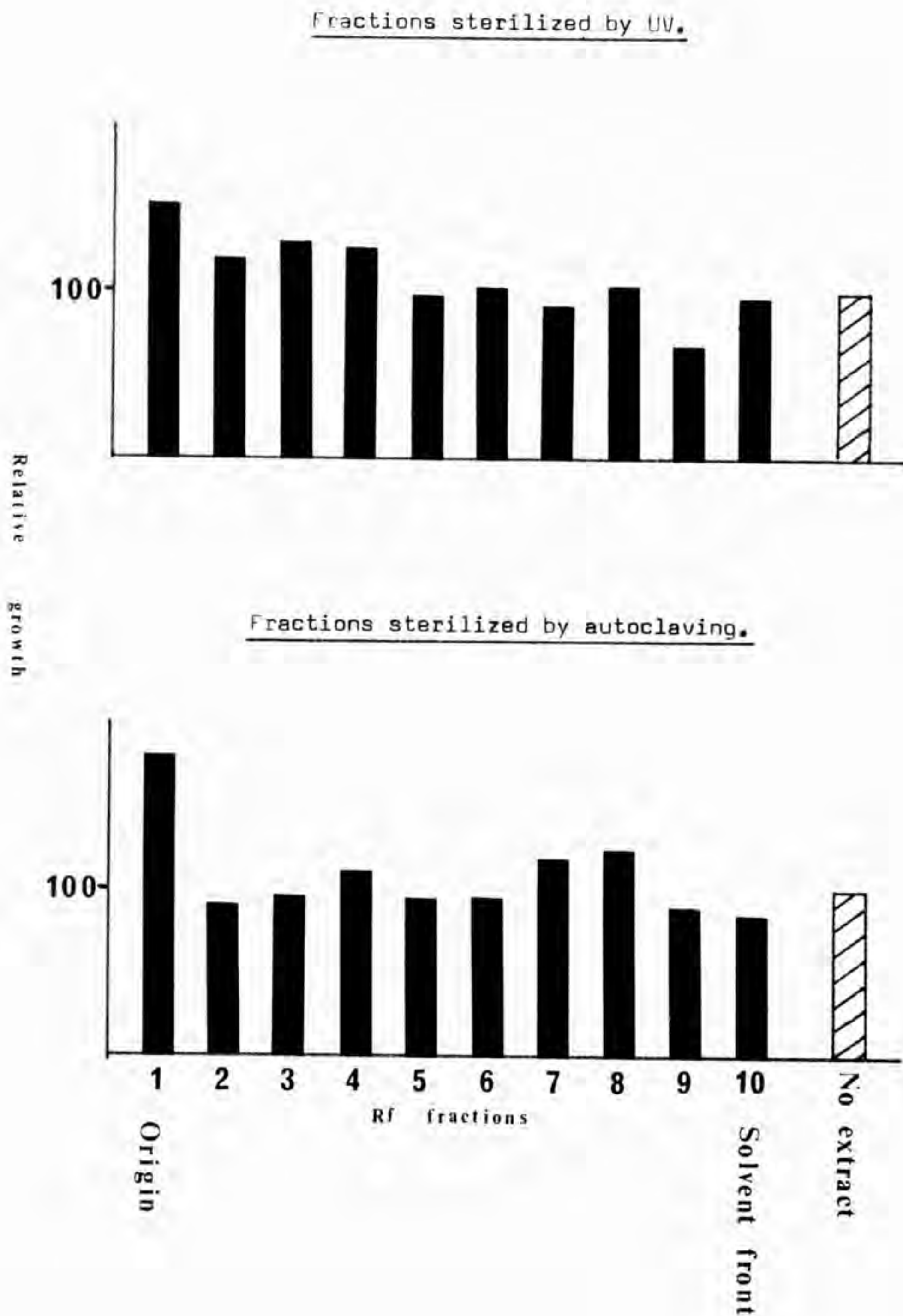


Fig. 44. Growth of M13, in MLM, with different Rf fractions, after the fractionation of excised tomato root extract in BuP, by paper chromatography.

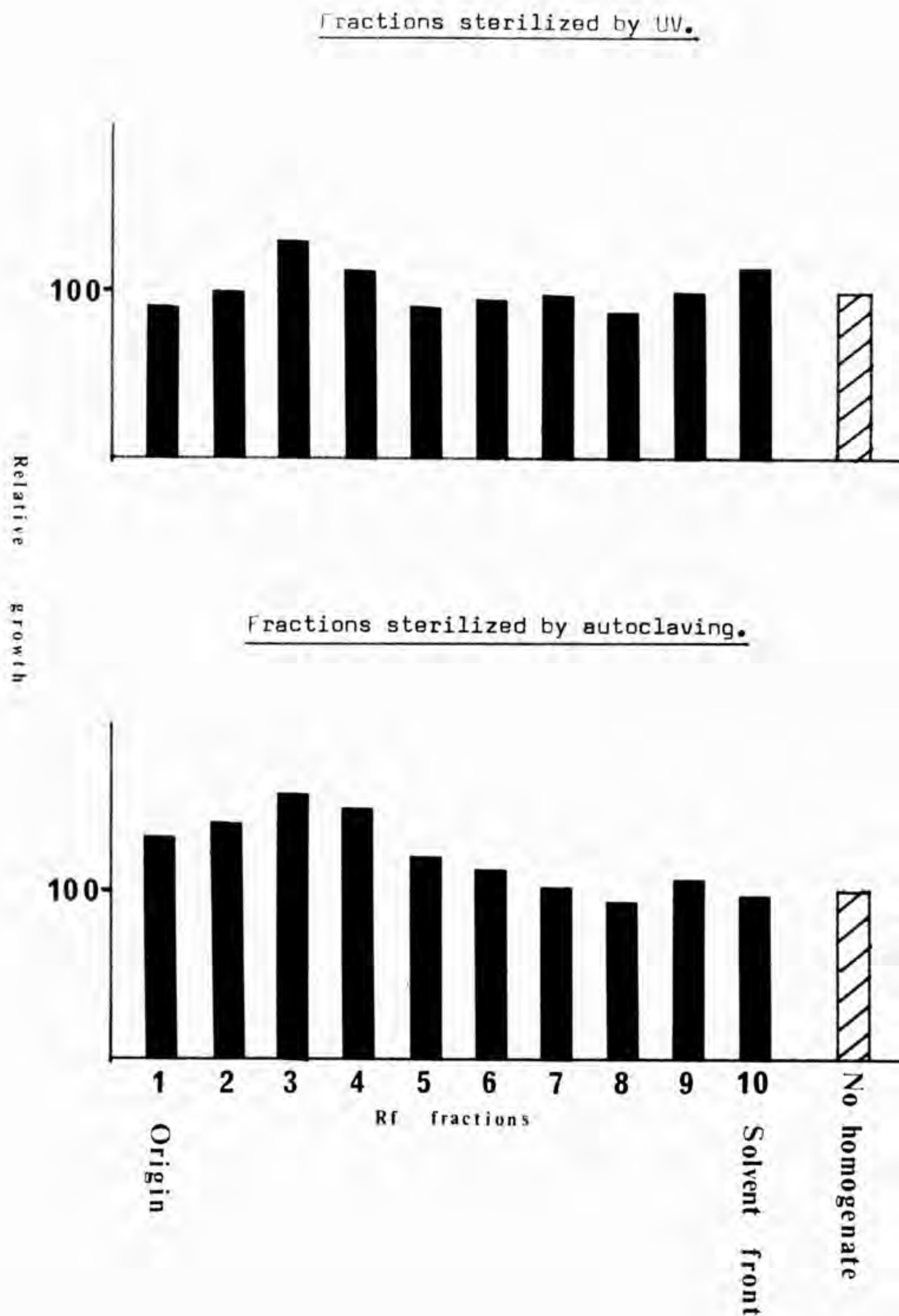


Fig. 45. Growth of M13, in MLM, with different Rf fractions, after the fractionation of excised tomato root homogenate in BuA, by paper chromatography.

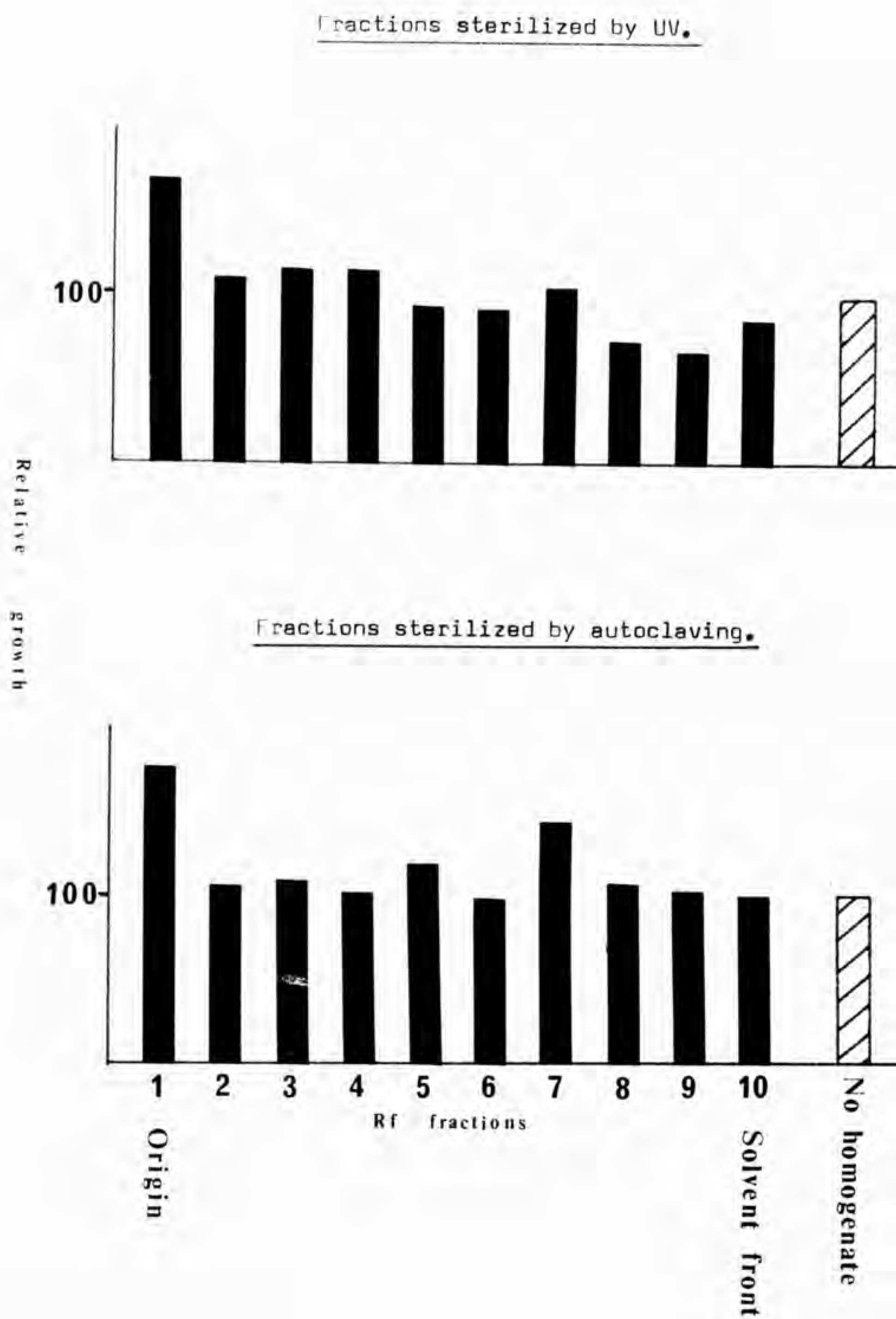


Fig. 46. Growth of M13, in MLM, with different Rf fractions, after the fractionation of excised tomato root homogenate in BuP, by paper chromatography.

Consistent patterns of growth stimulation were also obtained after fractionation of root preparations in BuP (see Figs. 41, 44 and 46). The peak growth stimulatory Rf fractions after sterilizing under UV light were 1, 3 and 9 for live root exudate and 1 for extract. Fractionated homogenate had no significant peak Rf fraction, although the growth curve was similar in shape to the other excised tomato root preparations. After autoclaving, the growth stimulatory Rf fractions remained the same for extract, but became insignificant for live root exudate. However four specific Rf fractions which produced growth stimulation (1, 2, 5 and 7) were observed after autoclaving the fractionated homogenate.

Therefore the effect of autoclaving these Rf fractions (from live excised tomato root preparations, fractionated in BuA and BuP) varies, although in most cases (except extract fractionated in BuP), more growth stimulation was produced.

The growth of Boletus variegatus 1 with fractionated live root exudate, extract and homogenate of excised tomato roots, in BuA and BuP are given in Figs. 47 to 52 (also see Appendix D).

The Rf fractions of all fractionated root preparations inhibited the growth of Boletus variegatus 1, when fractionated in either BuA or BuP solvents (Figs. 47 to 52). Autoclaving reduced the general inhibition of most Rf fractions. There was some similarity between some of the growth curves of Boletus variegatus 1 for the various root preparations, less inhibition occurring at

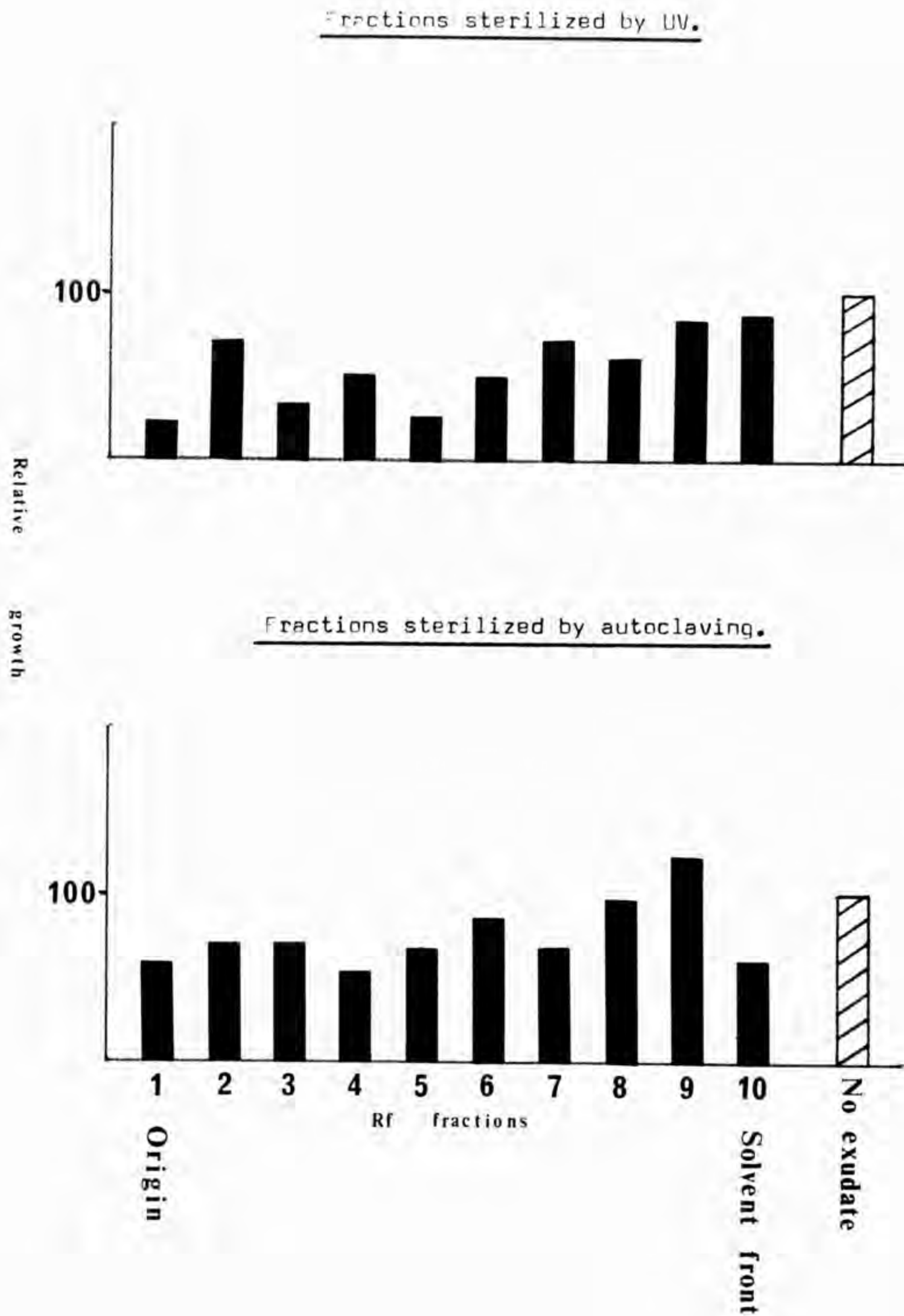


Fig. 47. Growth of Boletus variegatus 1 in MLM, with different Rf fractions, after the fractionation of live excised tomato root exudate in BuA, by paper chromatography.

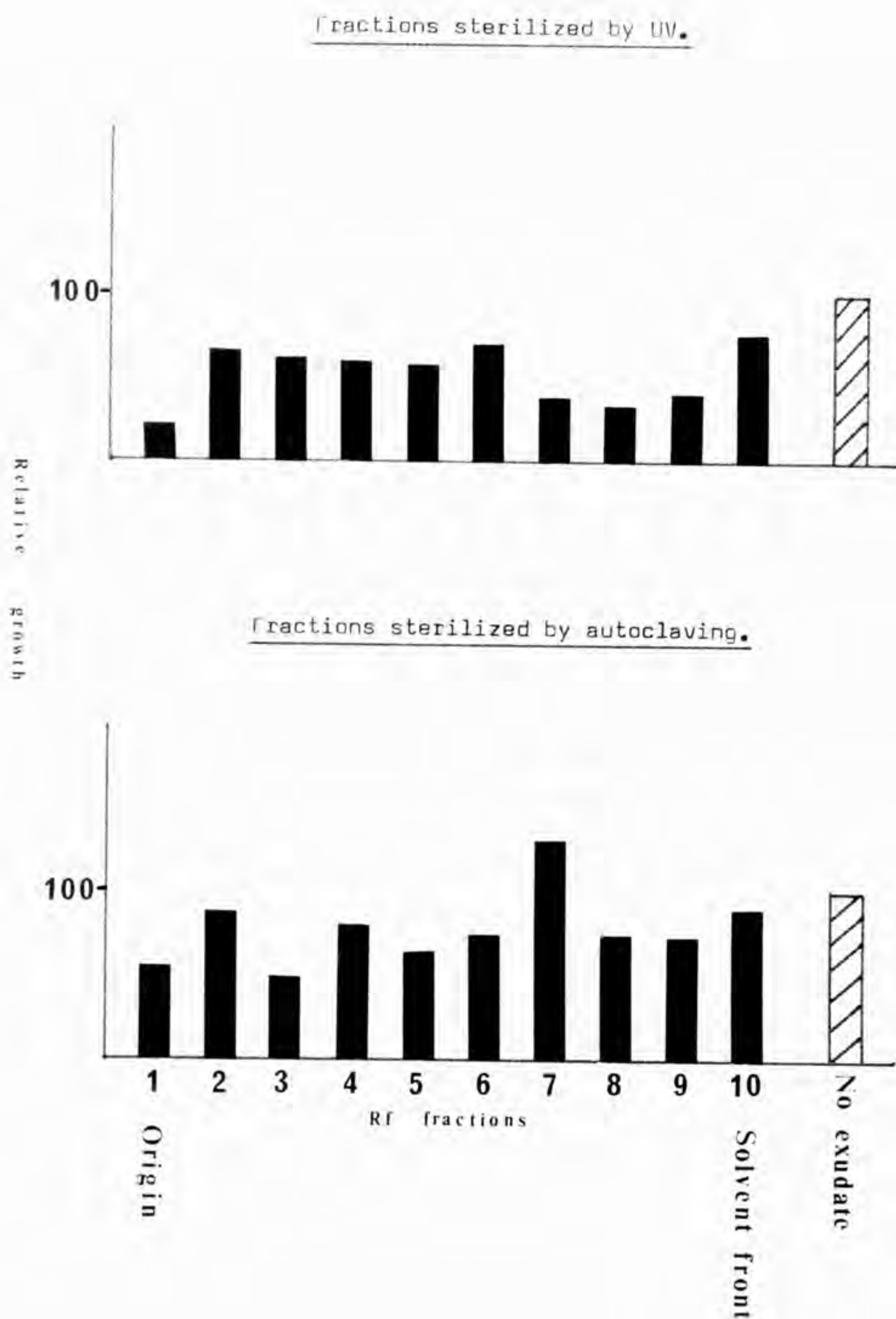


Fig. 48. Growth of Boletus variegatus 1, in MLM, with different Rf fractions, after the fractionation of live excised tomato root exudate in BuP, by paper chromatography.

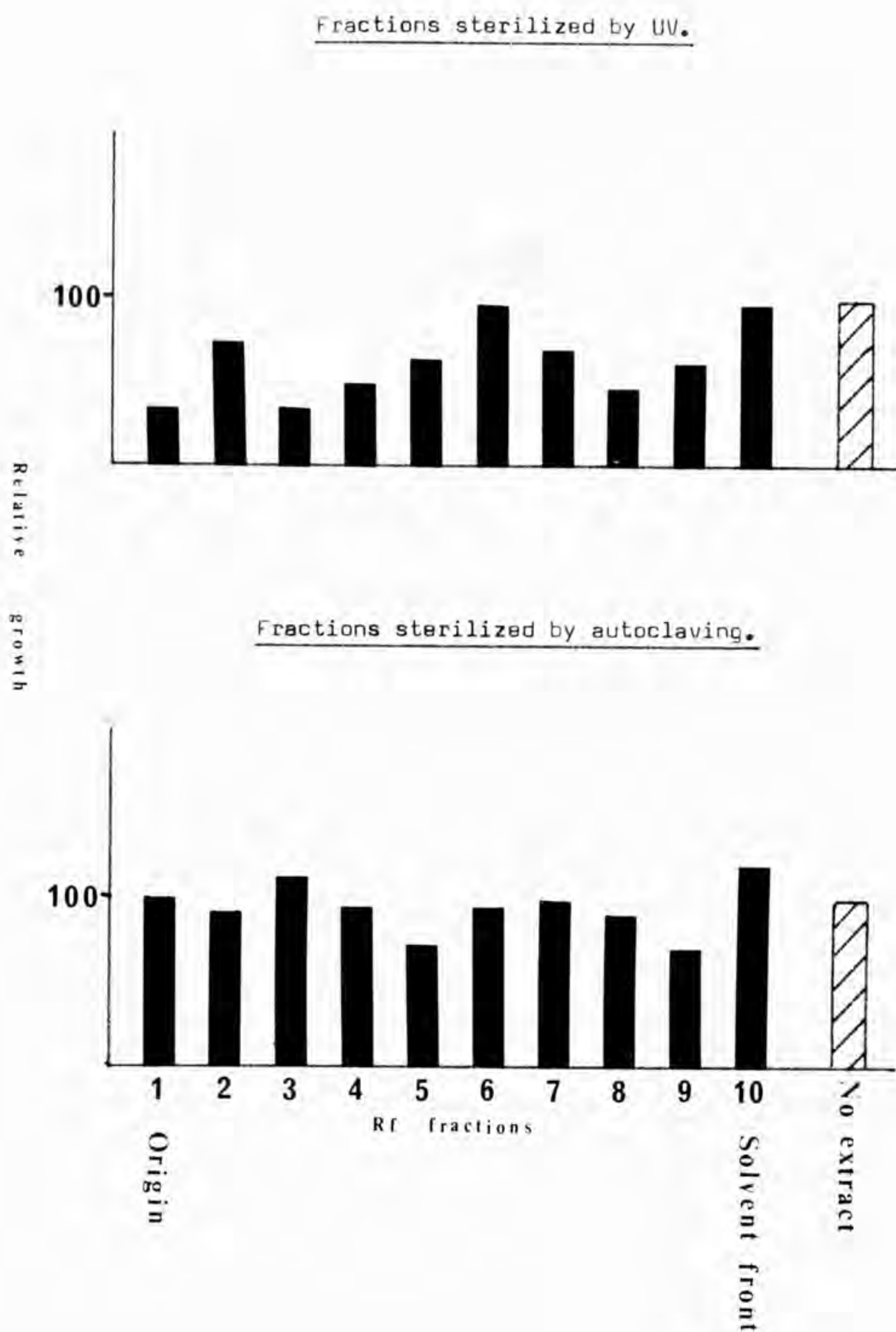


Fig. 49. Growth of Boletus variegatus 1, in MLM, with different Rf fractions, after the fractionation of excised tomato root extract in BuA, by paper chromatography.

Fractions sterilized by UV.

100

Relative growth

Fractions sterilized by autoclaving.

100

1 2 3 4 5 6 7 8 9 10

Rf fractions

Origin

Solvent front

No extract

Fig. 50. Growth of Boletus variegatus 1, in MLM, with different Rf fractions, after the fractionation of excised tomato root extract in BuP, by paper chromatography.

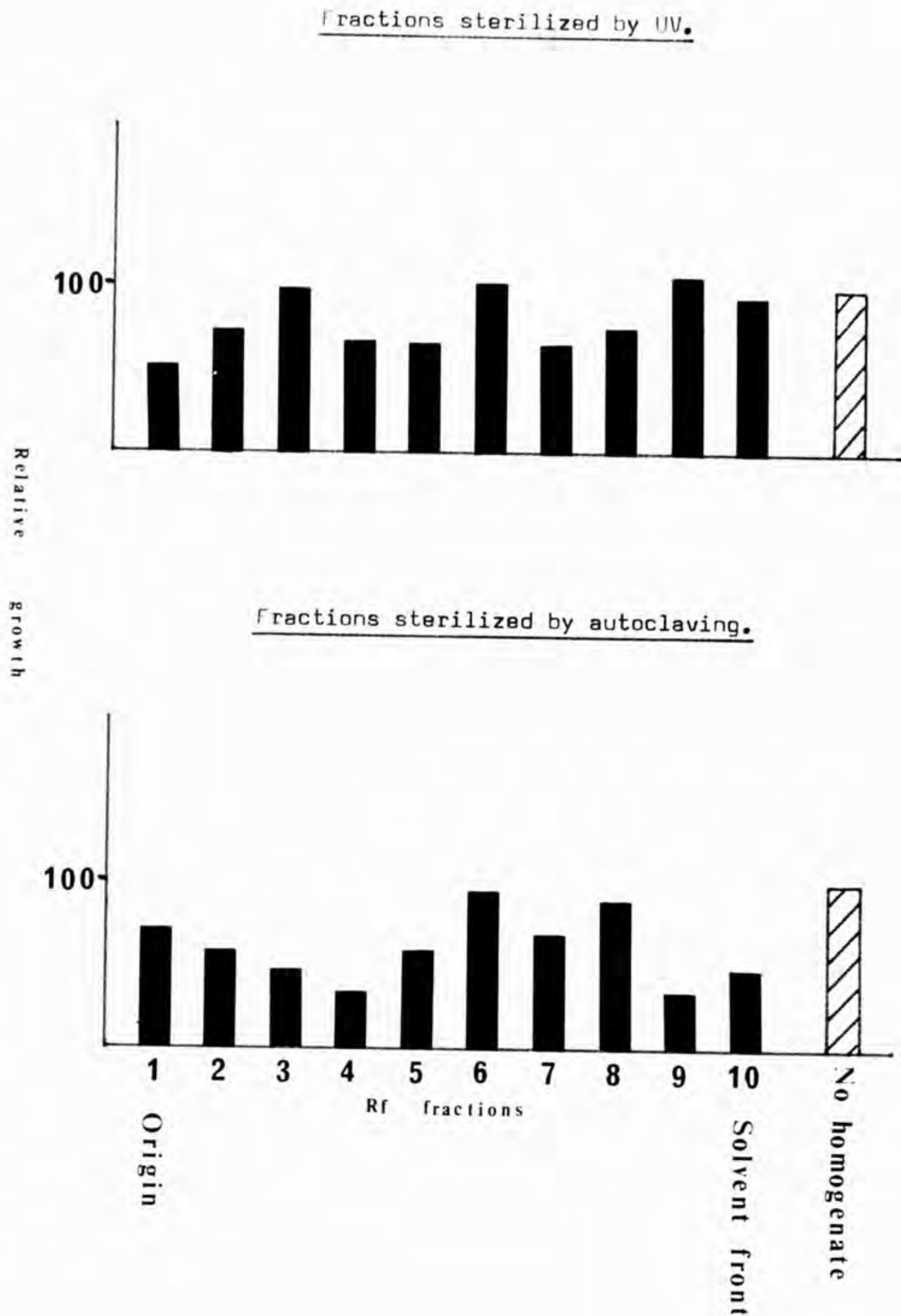


Fig. 51. Growth of Boletus variegatus 1, in MLM, with different Rf fractions, after the fractionation of excised tomato root homogenate in BuA, by paper chromatography.

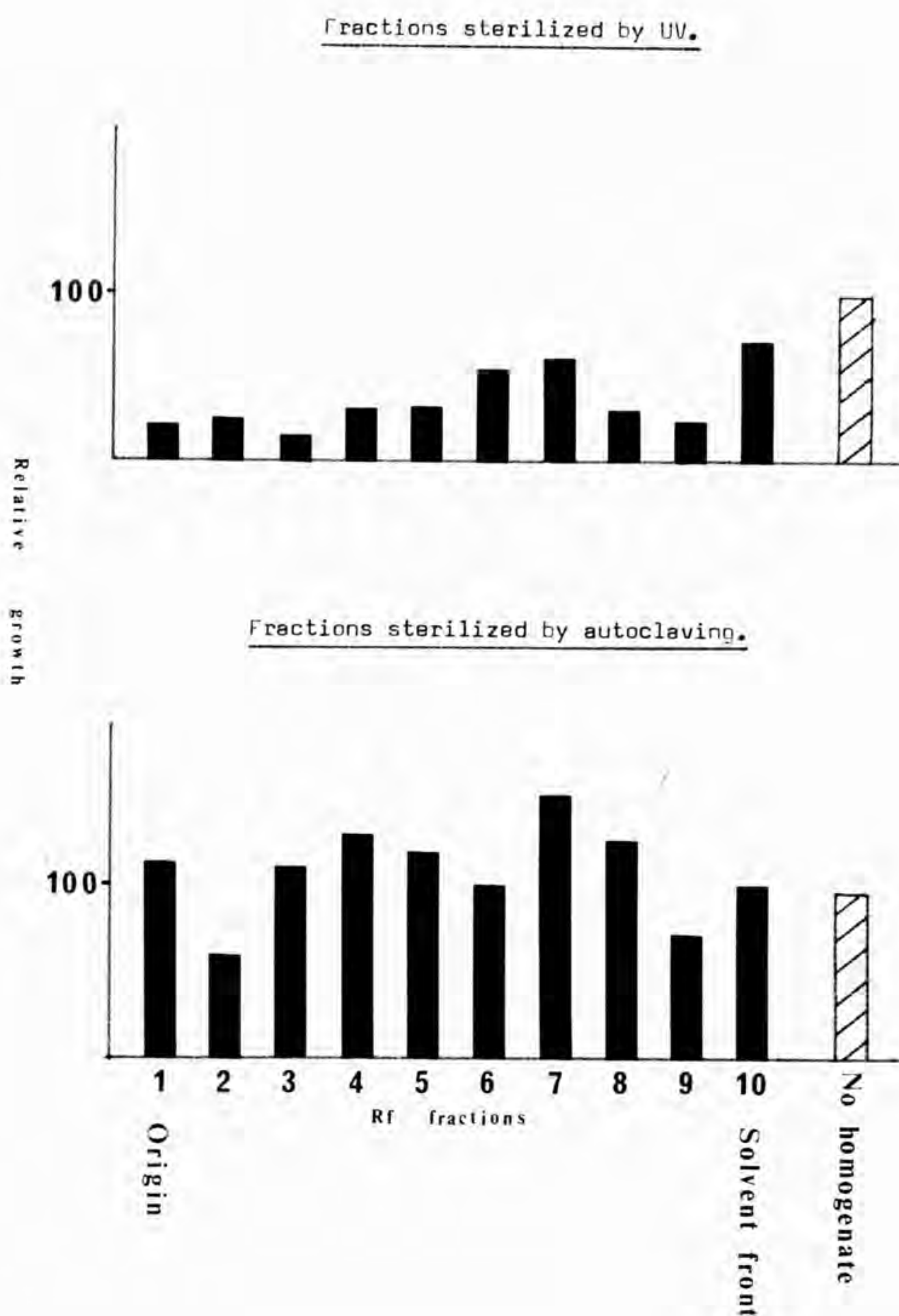


Fig. 52. Growth of Boletus variegatus 1, in MLM, with different Rf fractions, after the fractionation of excised tomato root homogenate in BuP, by paper chromatography.

Rf fractions 2-3, 6-9, and 9-10 in BuA. In BuP, peaks were not clearly defined or constant.

EXPERIMENTS WITH PINE SEEDLING ROOTS.

1. Experiments with pine seedling root exudates, extracts and homogenates in MLM.

The results of experiments with pine seedling root exudate, extract and homogenate from pine seedling roots grown for 3 weeks and 25 weeks in growth tubes are given in Tables 34 and 35 and Figs. 53 and 54.

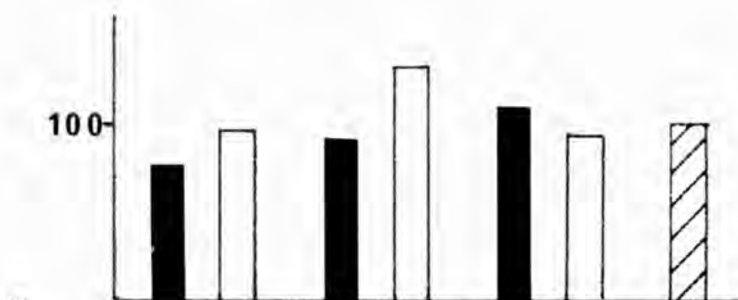
Table 34.

Growth of M13 in the presence of pine seedling root exudate, extract and homogenate in MLN.

Period of root exudation	Method of sterilization	Treatment	Mean dry weight, mg	Standard error	P
3 weeks	-	No root preparation (control)	124•[illegible]	(1•7)	-
3 weeks	Micropore filtration	Exudate	95•9	(10•7)	0•01*
3 weeks	Autoclaving	Exudate	121•4	(24•5)	n.s.
3 weeks	Micropore filtration	Extract	114•5	(9•2)	n.s.
3 weeks	Autoclaving	Extract	165•2	(45•8)	n.s.
3 weeks	Micropore filtration	Homogenate	136•2	(41•0)	n.s.
3 weeks	Autoclaving	Homogenate	120•2	(23•9)	n.s.
25 weeks	-	No root preparation (control)	20•6	(4•8)	-
25 weeks	Micropore filtration	Exudate	91•1	(6•5)	0•001
25 weeks	Autoclaving	Exudate	36•2	(2•0)	0•01
25 weeks	-	No root preparation (control)	114•5	(20•2)	-
25 weeks	Micropore filtration	Extract	194•3	(22•5)	0•01
25 weeks	Autoclaving	Extract	239•4	(54•8)	0•01
25 weeks	Micropore filtration	Homogenate	184•2	(12•4)	0•002
25 weeks	Autoclaving	Homogenate	224•9	(29•7)	0•002

* Inhibition

Pine seedling root preparations from seedlings grown for 3 weeks in growth tubes.



Relative growth

Pine seedling root preparations from seedlings grown for 25 weeks in growth tubes.

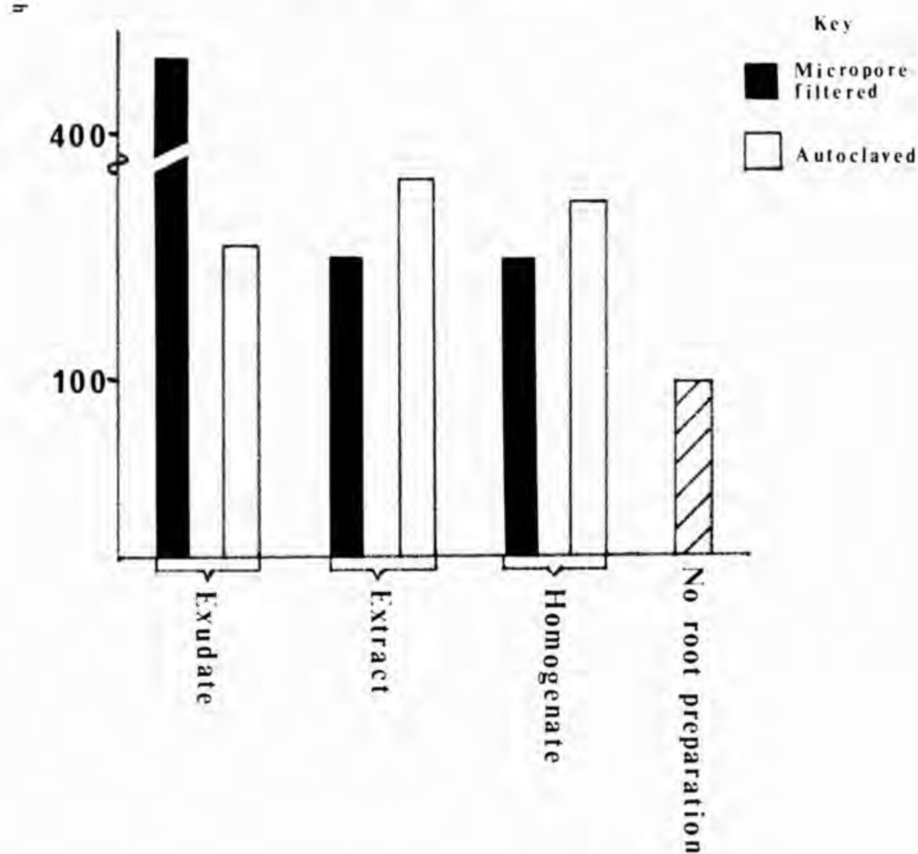


Fig. 53. Growth of M13 in the presence of pine seedling root exudate, extract and homogenate in MLM.

Table 35.

Growth of Boletus variegatus 1 in the presence of pine seedling root exudate, extract and homogenate in MLN.

Period of root exudation	Method of sterilization	Treatment	Mean dry weight, mg	Standard error	P
3 weeks	-	No root preparation (control)	6•3	(1•3)	-
3 weeks	Micropore filtration	Exudate	5•6	(2•9)	n.s.
3 weeks	Autoclaving	Exudate	8•8	(3•3)	n.s.
3 weeks	Micropore filtration	Extract	8•5	(2•8)	n.s.
3 weeks	Autoclaving	Extract	10•1	(2•1)	0•02
3 weeks	Micropore filtration	Homogenate	7•5	(2•9)	n.s.
3 weeks	Autoclaving	Homogenate	7•7	(4•0)	n.s.
25 weeks	-	No root preparation (control)	10•4	(4•3)	-
25 weeks	Micropore filtration	Exudate	0•3	(0•0)	0•01*

* Inhibition

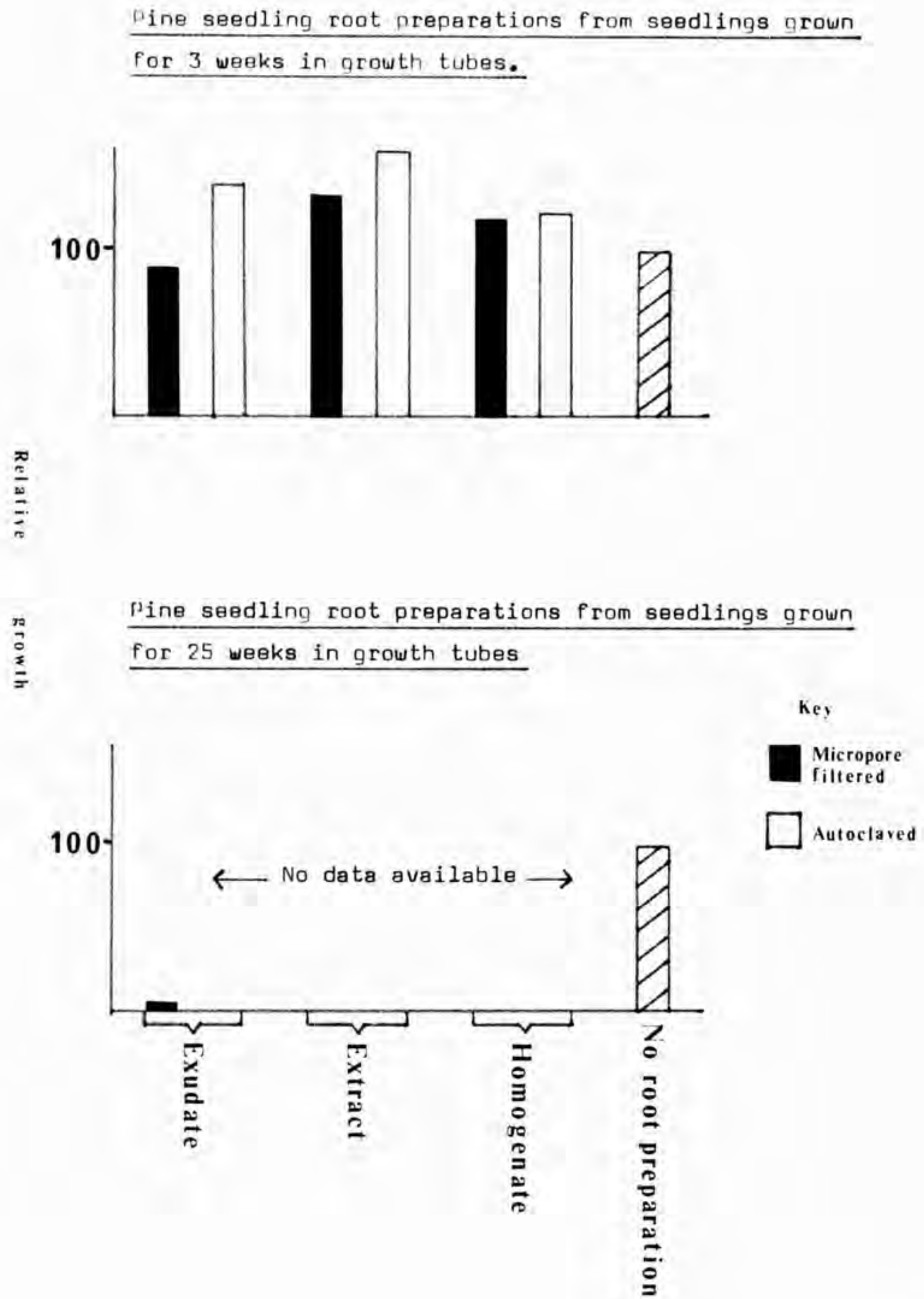


Fig. 54. Growth of Boletus variegatus 1 in the presence of pine seedling root exudate, extract and homogenate in MLM.

From Fig. 53, the growth response of M13 to the pine seedling root exudate (collected for 25 weeks) and extract and homogenate of these roots was very similar to that obtained with excised tomato root preparations (see Fig. 32), except that pine seedling root exudate stimulated more than extract or homogenate from these roots. However the growth response of M13 to micropore filtered pine seedling root exudate was very different to that produced by Boletus variegatus 1 (Fig. 54), since this root preparation inhibited the growth of B. variegatus1.

Micropore filtered pine seedling root exudate (from pine seedlings grown in growth tubes for 3 weeks) significantly inhibited the growth of M13, no root preparation from these pine seedlings causing growth stimulation. Only autoclaved pine seedling root extract significantly stimulated the growth of B. variegatus 1.

2. Chromatographic bioassay of pine seedling root exudates in MLM.

The results of fractionating pine seedling root exudate by paper chromatography using the BuA solvent and the effect on the growth of M13 and Boletus variegatus 1 are shown in Figs. 55 to 58 (also see Appendix D).

Significant growth stimulation of M13 occurred at Rf fractions 1 and 5 for exudate collected for 25 weeks (see Fig. 56) and at 2 for exudate collected for 3 weeks (see Fig. 55).

Pine seedling root exudate, like excised tomato root exudate, inhibited the growth of Boletus variegatus 1 at

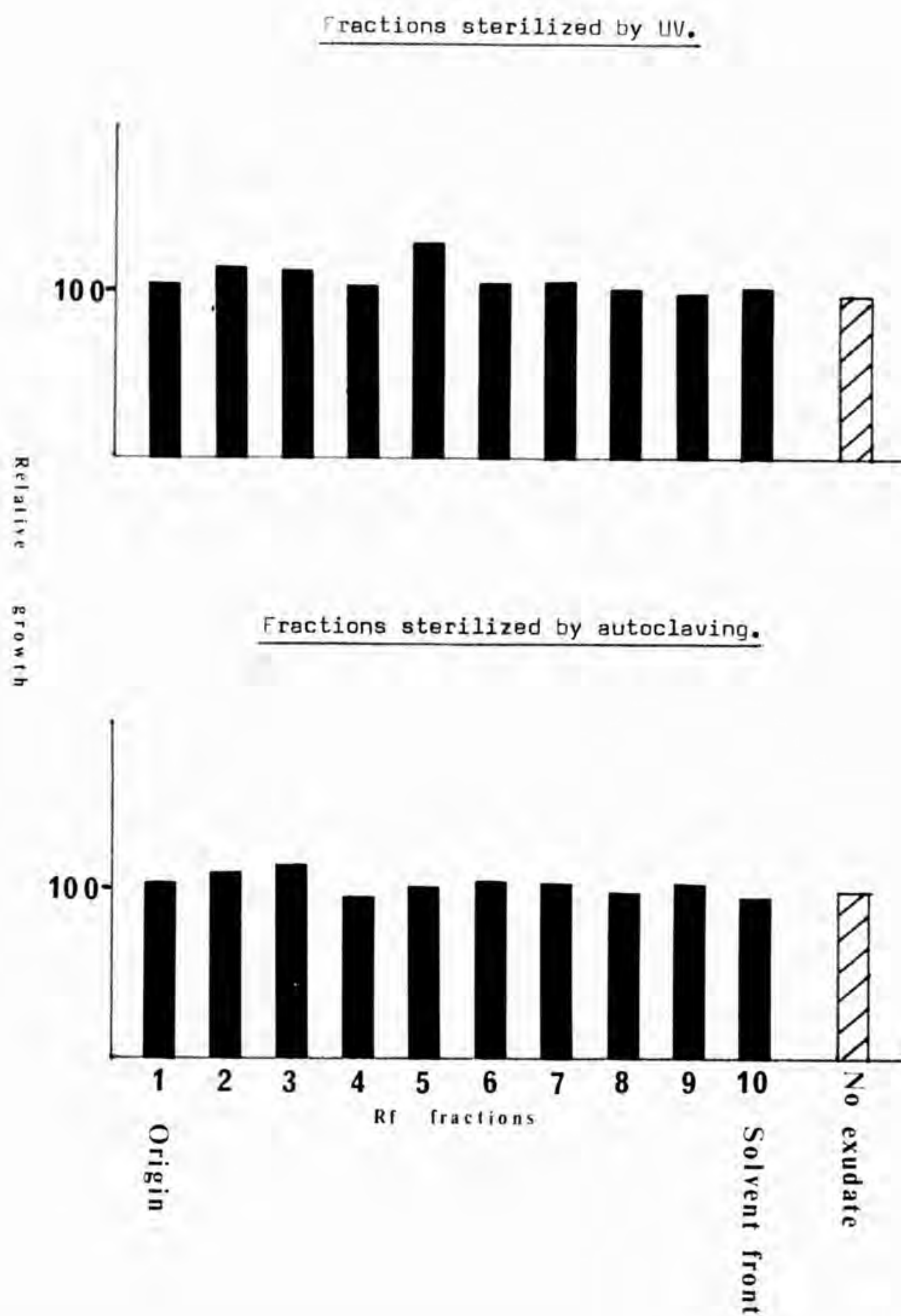


Fig. 55. Growth of M13, in MLM, with different Rf fractions, after the fractionation of pine seedling root exudate (collected for 3 weeks) in BuA, by paper chromatography.

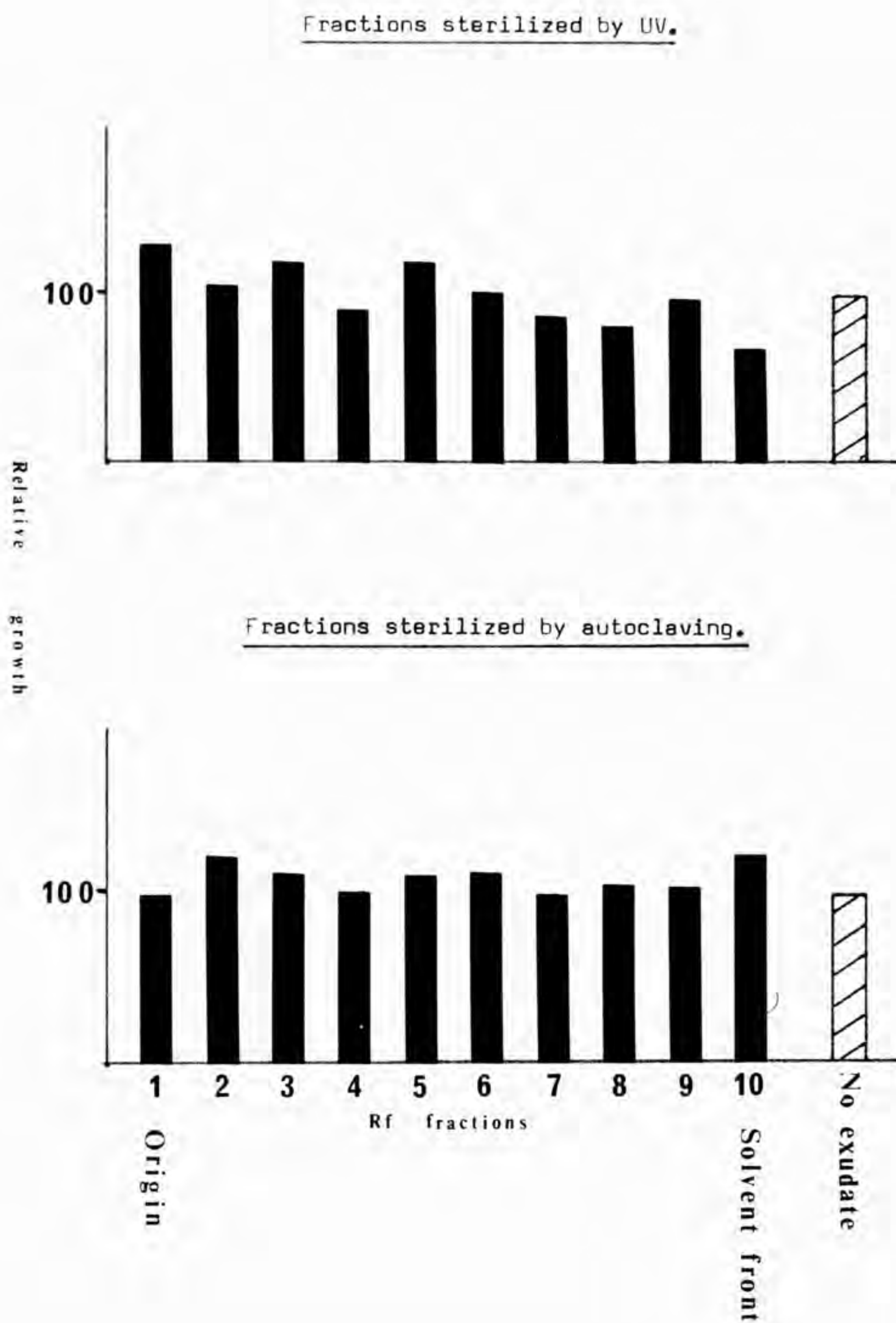


Fig. 56. Growth of M13, in MLM, with different Rf fractions, after the fractionation of pine seedling root exudate (collected for 25 weeks) in BuA, by paper chromatography.

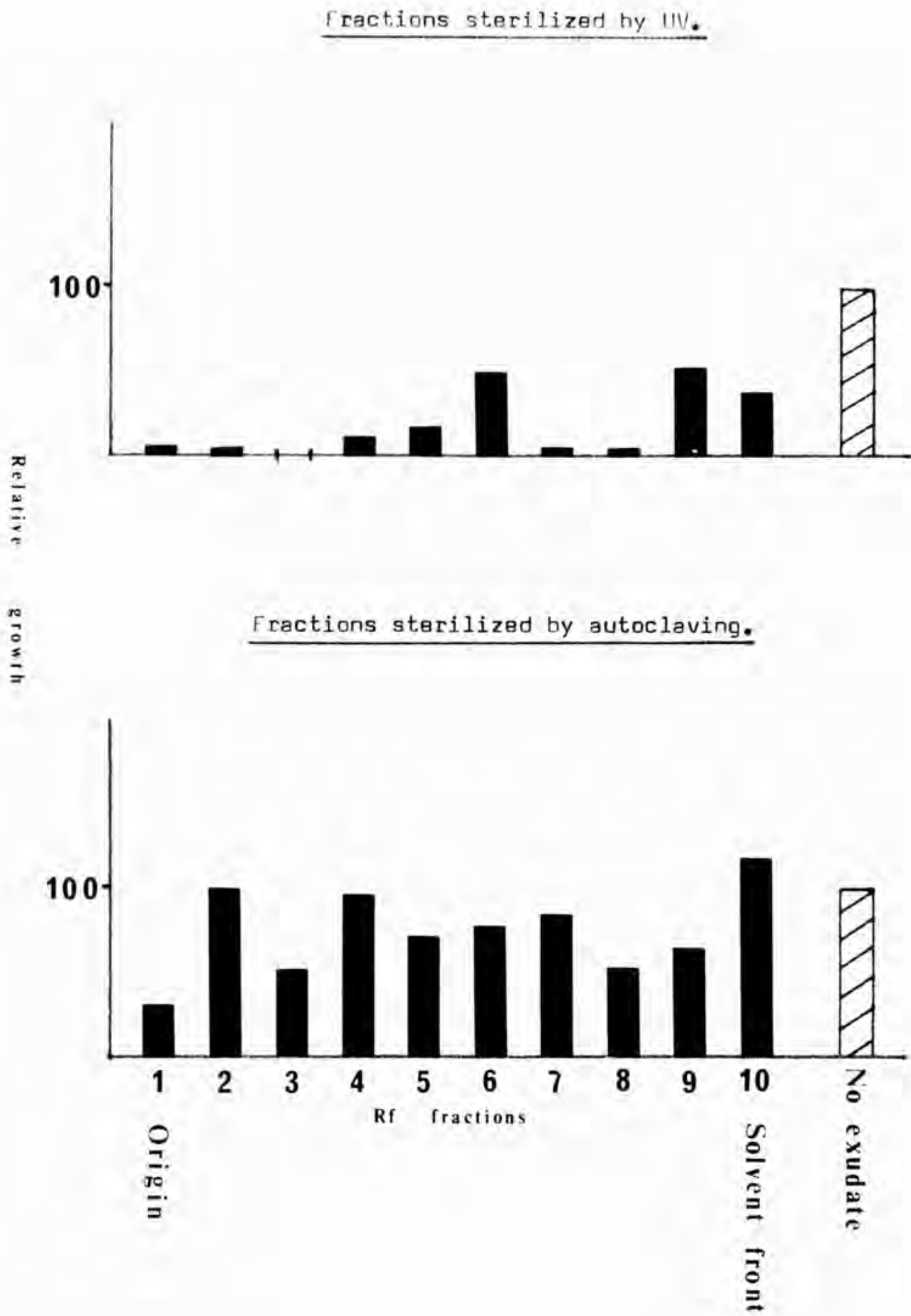


Fig. 57. Growth of Boletus variegatus 1, in MLM, with different Rf fractions, after the fractionation of pine seedling root exudate (collected for 3 weeks) in BuA, by paper chromatography.

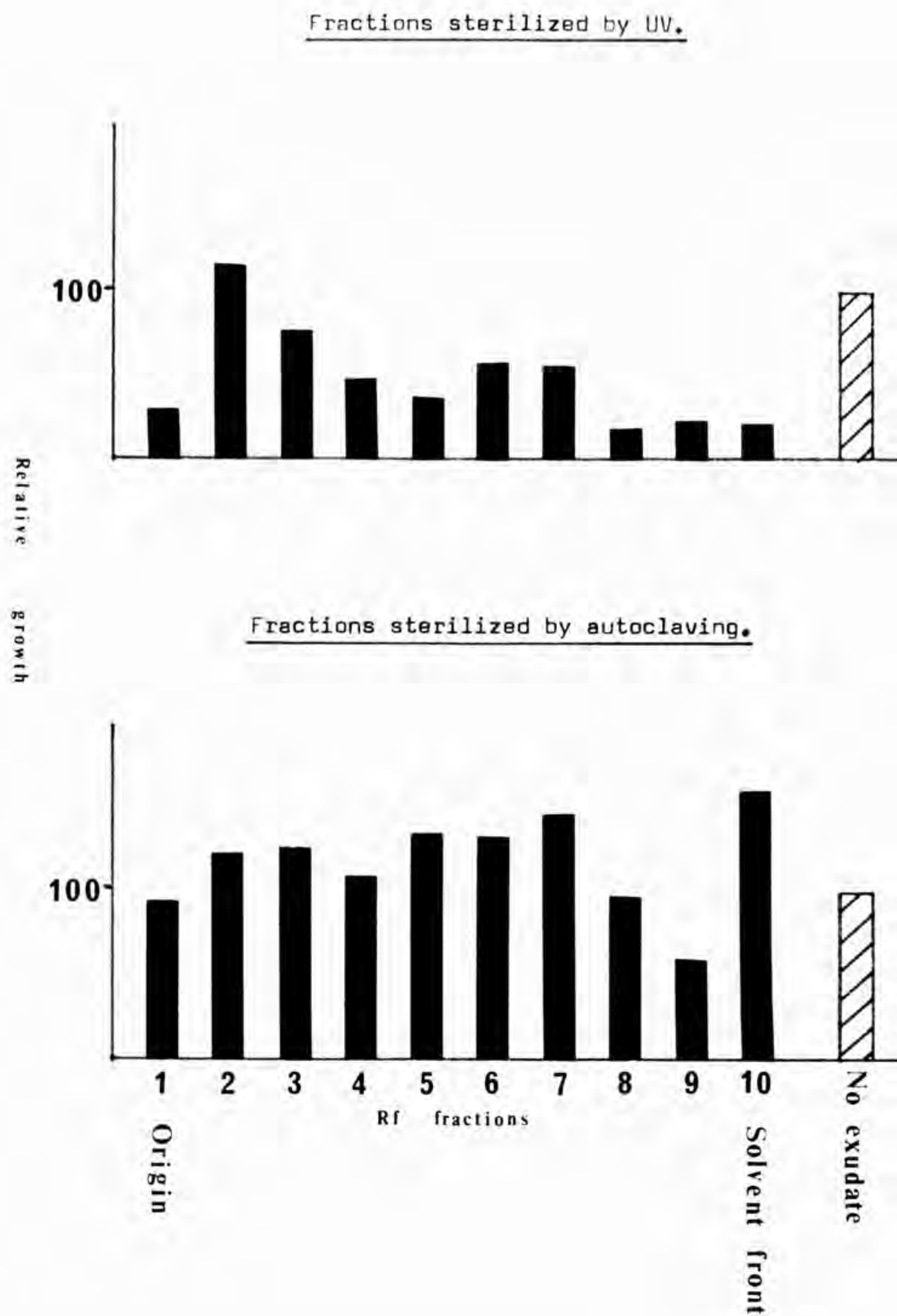


Fig. 58. Growth of Boletus variegatus 1, in MLM, with different Rf fractions, after the fractionation of pine seedling root exudate (collected for 25 weeks) in BuA, by paper chromatography.

most Rf fractions and this was reduced after autoclaving, although no significant growth stimulation occurred. (See Figs. 57 and 58).

A summary of the results of the chromatographic bioassay of excised tomato root preparations and pine seedling root exudates to show the stimulatory Rf fractions in BuA and BuP are shown below (Table 36).

Table 36.

A summary of M13 and Boletus variegatus1 growth stimulatory Rf fractions in fractionated excised tomato root exudate (from live roots collected in distilled water), extract, homogenate and dialysate (from live exuding roots enclosed in dialysis membrane bags and collected in distilled water) and pine seedling root exudates.

i. Excised tomato root preparations fractionated in BuA.

Fungus	Method of sterilization	Stimulatory Rf fractions of root preparations			
		Exudate	Extract	Homogenate	Dialysate
M13	UV	3,4,6	1,2,3,4 8,10	3,10	1,2,3,4 5,6,7,8
M13	Autoclaving	1,2,3,4 5,6,7,8	2,3,4,5	1,2,3,4	-
B. variegatus 1	UV	None	None	None	-
B. variegatus 1	Autoclaving	None	None	None	-

Continued .....

Table 36. (cont.)

ii. Excised tomato root preparations fractionated in BuP.

Fungus	Method of sterilization	Stimulatory Rf fractions of root preparations		
		Exudate	Extract	Homogenate
M13	UV	1,3,9	1	None
M13	Autoclaving	None	1	1,2,5,7
B. variegatus 1	UV	None	None	None
B. variegatus 1	Autoclaving	None	None	None

iii. Pine seedling root exudates fractionated in BuA.

Fungus	Period of collection of exudate	Method of sterilization	Stimulatory Rf fractions of exudate
M13	3 weeks	UV	2
M13	3 weeks	Autoclaving	None
B. variegatus 1	3 weeks	UV	None
B. variegatus 1	3 weeks	Autoclaving	None
M13	25 weeks	UV	1,5
M13	25 weeks	Autoclaving	None
B. variegatus 1	25 weeks	UV	None
B. variegatus 1	25 weeks	Autoclaving	None

CHROMATOGRAPHY OF EXCISED TOMATO ROOT EXUDATE (FROM LIVE ROOTS COLLECTED IN DISTILLED WATER), EXTRACT AND HOMOGENATE.

In order to characterize the stimulatory factor(s), which stimulates the growth of M13, chromatograms were sprayed to try to locate, in excised tomato root preparations, certain groups of substances known to occur generally in plant root exudates.

1. Amino acids.

These were all tentatively identified by comparison with marker amino acids as shown in Table 37.

Table 37.

Amino acids present in live excised tomato root exudate, extract and homogenate.

Amino acids	Presence in root preparation			Rf values (by ascending chromatography)		Rf equivalent to fractions used in chromatographic bioassay	
	Exudate	Extract	Homogenate	BuA	PhAm	BuA	BuP
Isoleucine	+	+	+	6•2	8.3	7	6-7
Methionine or valine	+	+	+	5•1	7•9	6	6
Alanine	-	+	+	2•6	5•8	4	4
Lysine or histidine	+	-	+	1•2	7•5	3	2-3
Glutamine	+	+	+	1•5	5•3	3	3
*Asparagine	+	-	+	1•0	3•6	2	2-3
Serine	+	+	+	1•4	3•2	3	4
Aspartic acid	+	+	+	1•9	2•5	3	3
Cysteine	-	+	+	1•2	1•3	2	2-3
Unknown 1	+	-	+	4•0	7•9	5	7

* Orange spot in ninhydrin

Key

PhAm = Phenol - ammonia - water (160:1:40)

BuA = Butanol - acetic acid - water (60:15:25)

BuP = Butanol - pyridine - water (60:60:60)

2. Sugars.

These were tentatively identified by comparison with marker sugars. Glucose, fructose and sucrose were all present in excised tomato root extract and homogenate, but only glucose and fructose were present in live excised tomato root exudate. Rf and Rg values for these sugars in the solvents used were as follows (Table 38).

Table 38.

Rf and Rg values for the sugars present in live excised tomato root exudate, extract and homogenate.

Sugar	Colour in aniline-diphenylamine reagent	Rf equivalent to fractions used in chromatographic bioassay		Rg values in IPrAq (descending)
		BuA	BuP	
Glucose	Grey	3	all run	10•0
Fructose	Brown	4	together	10•6
Sucrose	Brown	2-3	at Rf 7-9	8•1

Key

BuA, BuP — See Table 37

IPrAq = Isopropanol - water (160:40)

3. Organic acids.

Two organic acids were located in live excised tomato root exudate and one in extract and homogenate, but they could not be equated with any marker organic acid (Table 39).

Table 39.

Unnamed organic acids present in live excised tomato root exudate, extract and homogenate.

Organic acid spot	Presence in root preparation			Rf equivalent to fractions used in chromatographic bioassay	
	Exudate	Extract	Homogenate	BuA	BuP
Unknown I	+	+	+	2	7
Unknown II	+	-	-	3-4	6

Key

BuA } See Table 37
BuP }

When these tomato root preparations were run descending in formic acid - amyl alcohol - chloroform - water solvent, four organic acid spots were located, two being tentatively identified as citric acid and tertaric acid, by comparison with marker organic acids. Also one basic spot occurred which ran only a short distance from the origin and which appeared blue in colour when sprayed with bromocresol green reagent (the organic acids forming yellow spots with this reagent) (see Table 40).

Table 40.

Organic acids present in live excised tomato root exudate, extract and homogenate when run descending in formic acid - amyl alcohol - chloroform - water solvent.

Organic acid	Type of spot (acidic or basic) in bromocresol green reagent	Rf value	Presence in root preparation		
			Exudate	Extract	Homogenate
Citric acid	Acidic	2·9	+	+	+
Tartaric acid	Acidic	2·1	+	+	+
Unknown A	Acidic	4·6	-	+	+
Unknown B	Acidic	0·8	+	+	+
Unknown C	Basic	0·3	+	+	+

4. Ehrlich-positive substances.

These were related where possible to marker Ehrlich-positive compounds, as in the following table (Table 41).

Table 41.

Ehrlich-positive substances present in live excised

Substance	Colour of spot in:-		Presence in root preparation		
	Ehrlich reagent	Long wave UV	Exudate	Extract	Homogenate
Tryptophan	Purple	Negative	+	+	+
Urea	Yellow	Negative	+	+	+
Unknown 1	Yellow	Blue	+	+	+
Unknown 2	Yellow	Blue	+	+	+
Unknown 3	Blue	Negative	+	-	+
Unknown 4	Pink	Negative	+	-	+
Unknown 5	Pink	Blue	+	+	+

Key

BuA, BuP } See Table 37

IPrAm = Isopropanol - ammonia - water (200:10:20)

tomato root exudate, extract and homogenate.

Rf equivalent to fractions used in chromatographic bioassay		Actual Rf values in:-	
BuA	BuP	IPrAm	BuA (descending)
5	6–7	Absent	4•1
Absent	5	3•7	Absent
1–2 }	The two spots run together at Rfs:-		
2–3 }	2–3	0•4	1•0
9 }	8–10 (only homogenate)	1•6	8•2
10 }		2•4 (only exudate)	Absent
4	7–8	1•0	3•0

From the above table, tryptophan and urea were tentatively identified as being present in the excised tomato root preparations.

5. Phenols and phenolic acids.

Chromatograms were run to determine whether phenols and phenolic acids were present in these samples, but no attempts were made to identify them (Tables 42 and 43).

Table 42.

Phenolic acids present in live excised tomato root exudate, extract and homogenate.

Phenolic acid	Colour in sulphanilic acid reagent	Presence in root preparation			Rf equivalent to fractions used in chromatographic bioassay BuA	Actual Rf values IPrAm
		Exudate	Extract	Homogenate		
Unknown 1	Light blue	+	-	-	3	Absent
Unknown 2	Pink	+	+	+	2 (Unknowns 2 and 3)	3•7
Unknown 3	Pink	+	+	+		1•8
Unknown 4	Yellow	+	+	+	Absent	5•4

Key

BuA = See Table 37

IPrAm = See Table 41

Table 43.

Phenols present in live excised tomato root exudate, extract and homogenate.

Phenol	Presence in root preparation			Rf equivalent to fractions used in chromatographic bioassay.	Actual Rf value
	Exudate	Extract	Homogenate	BuA	BuA
Unknown 1	+	+	+	1–2	1·0
Unknown 2	+	+	+	3	2·0
Unknown 3	+	+	+	3–4	3·0
Unknown 4	–	+	–	5–8	6·0

Key

BuA = See Table 37

Four phenolic acids were present in live excised tomato root exudate (see Table 42), but only three of these were present in excised tomato root extract and homogenate.

In excised tomato root extract there were four phenols present and three of these also occurred in the exudate and homogenate of excised tomato roots.

6. Tryptamines.

No further tryptamines were identified using the ninhydrin – acetic acid reagent, other than those already identified using the ninhydrin – acetone reagent (i.e. for amino acid identification).

7. Vitamins.

The only long wave, ultra-violet (UV) fluorescent vitamin in the excised tomato root exudate (from live roots), extract and homogenate, other than pyridoxine (see later results, page 264) was riboflavin (see Figs. 59 - 62). This vitamin gave rise to several spots after running in both BuA and BuP solvent systems. In all excised tomato root preparations these spots occurred at Rf fractions 1, 2, 3-4 and 7-8 in BuA, whereas in BuP the spots were at Rf fractions 1-2, 3 and 8 for homogenate, 1, 2, 4 and 8 for extract and 1, 2, 3 and 8 for the exudate. These spots correspond with the marker riboflavin in BuA, but in BuP the marker riboflavin only ran at Rf fractions 1 and 8.

These results do suggest that the unknown M13 growth stimulatory factor(s) could be riboflavin, for the Rf fractions for this vitamin are very similar to those found in live excised tomato root exudate, extract and homogenate where growth stimulation occurred in the two different solvent systems.

Riboflavin was also identified in pine seedling root exudate (collected for 3 weeks) at Rf fractions 1 and 4 in BuA and 1-3 and 8 in BuP. Hence this may also be the M13 growth stimulatory factor(s). Also it was noted that the pine seedling root exudate contained much more riboflavin (approximately 31 times) per milligram dry weight of root than excised tomato root preparations (see Figs. 63 and 64).

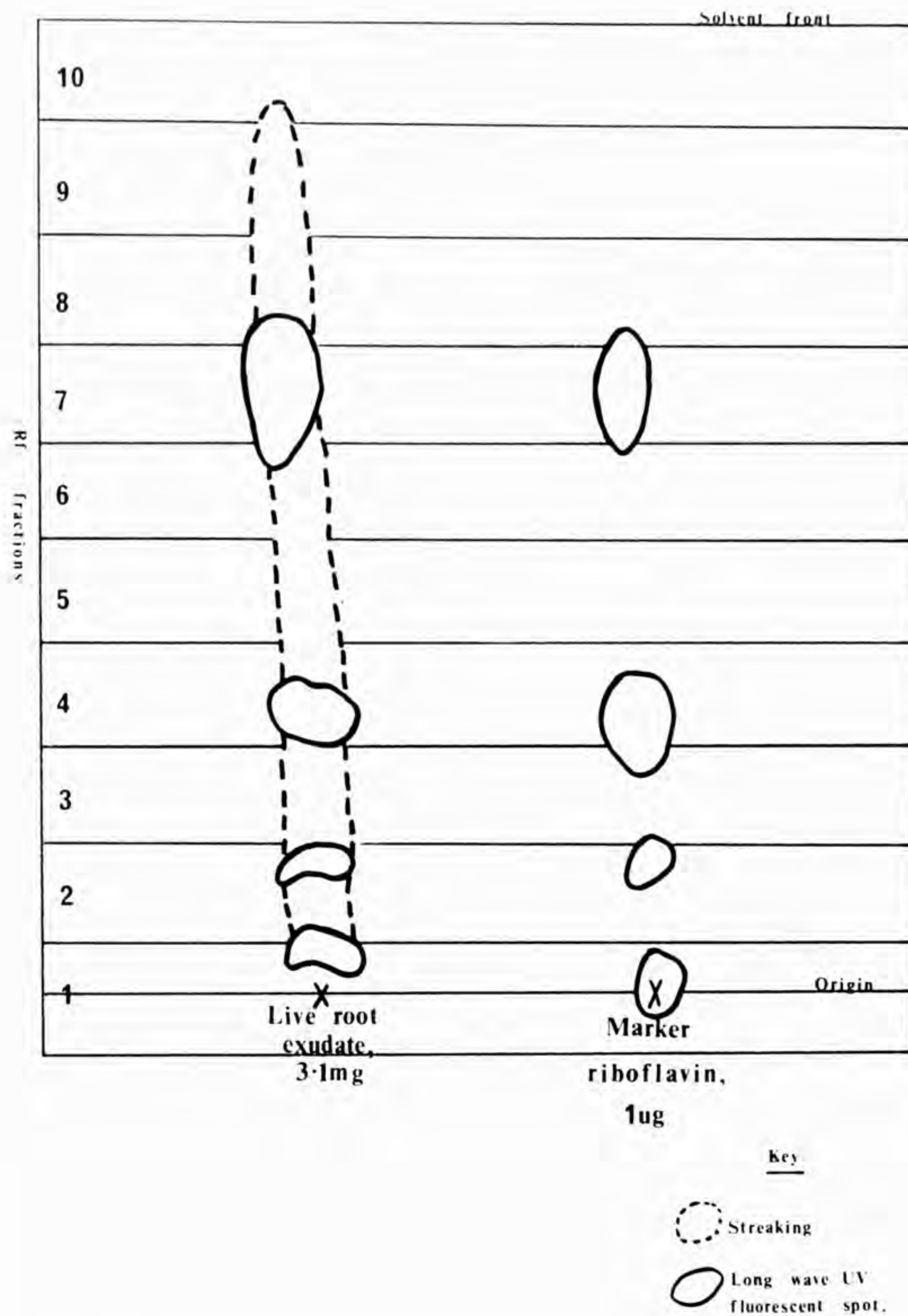


Fig. 59. Riboflavin present in live excised tomato root exudate after fractionation in BuA.

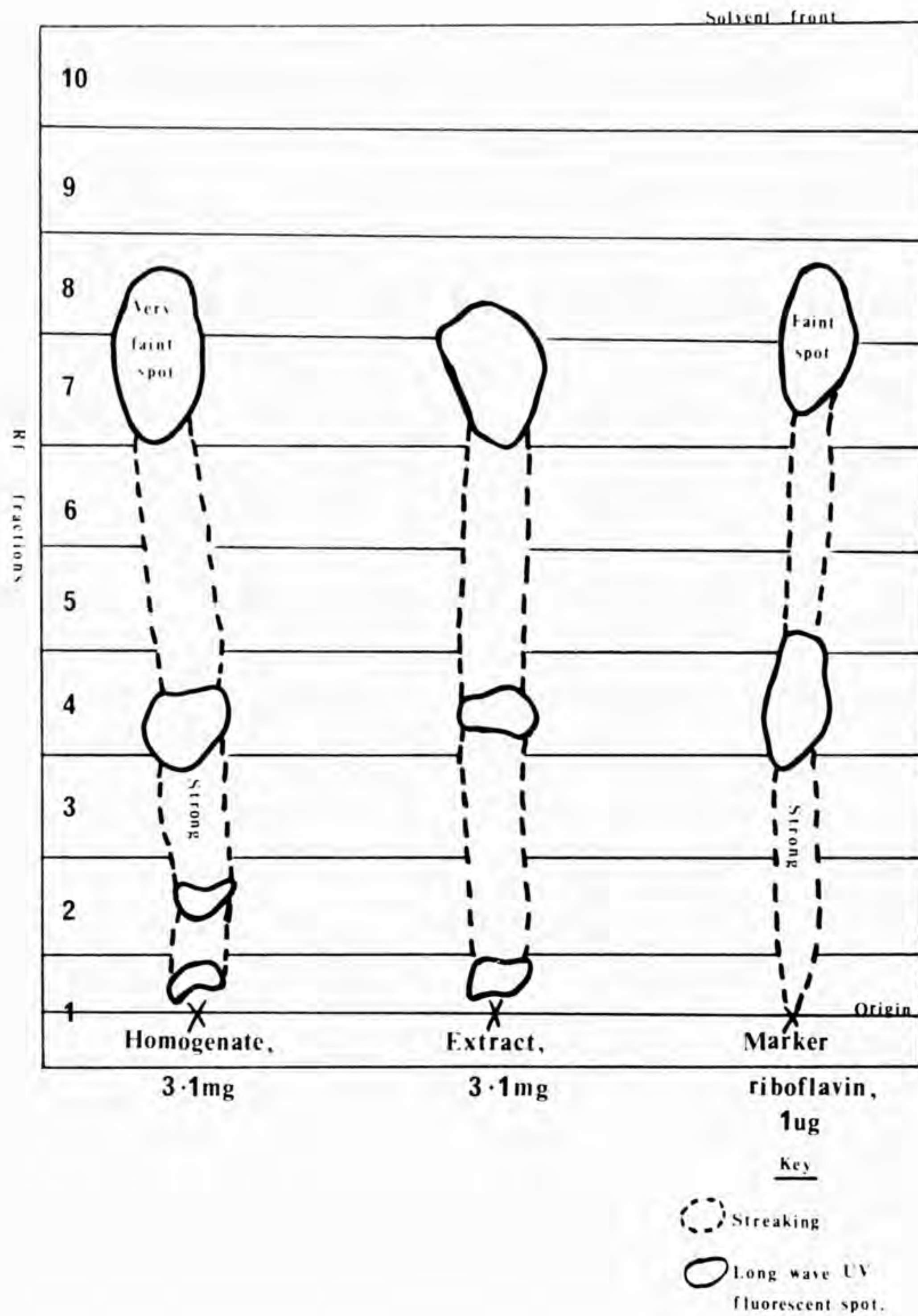


Fig. 60. Riboflavin present in excised tomato root extract and homogenate after fractionation in BuA.

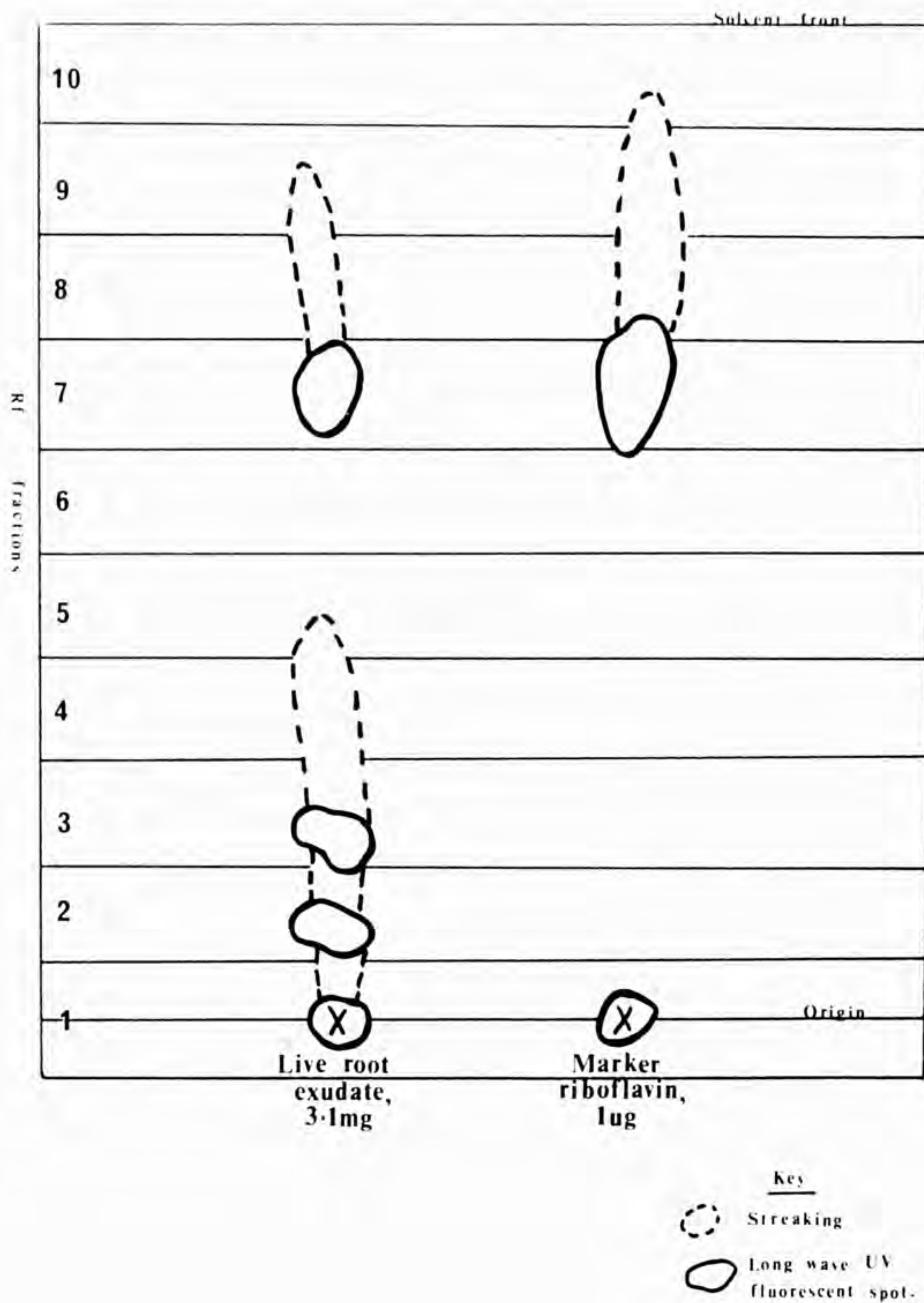


Fig. 61. Riboflavin present in live excised tomato root exudate after fractionation in BuP.

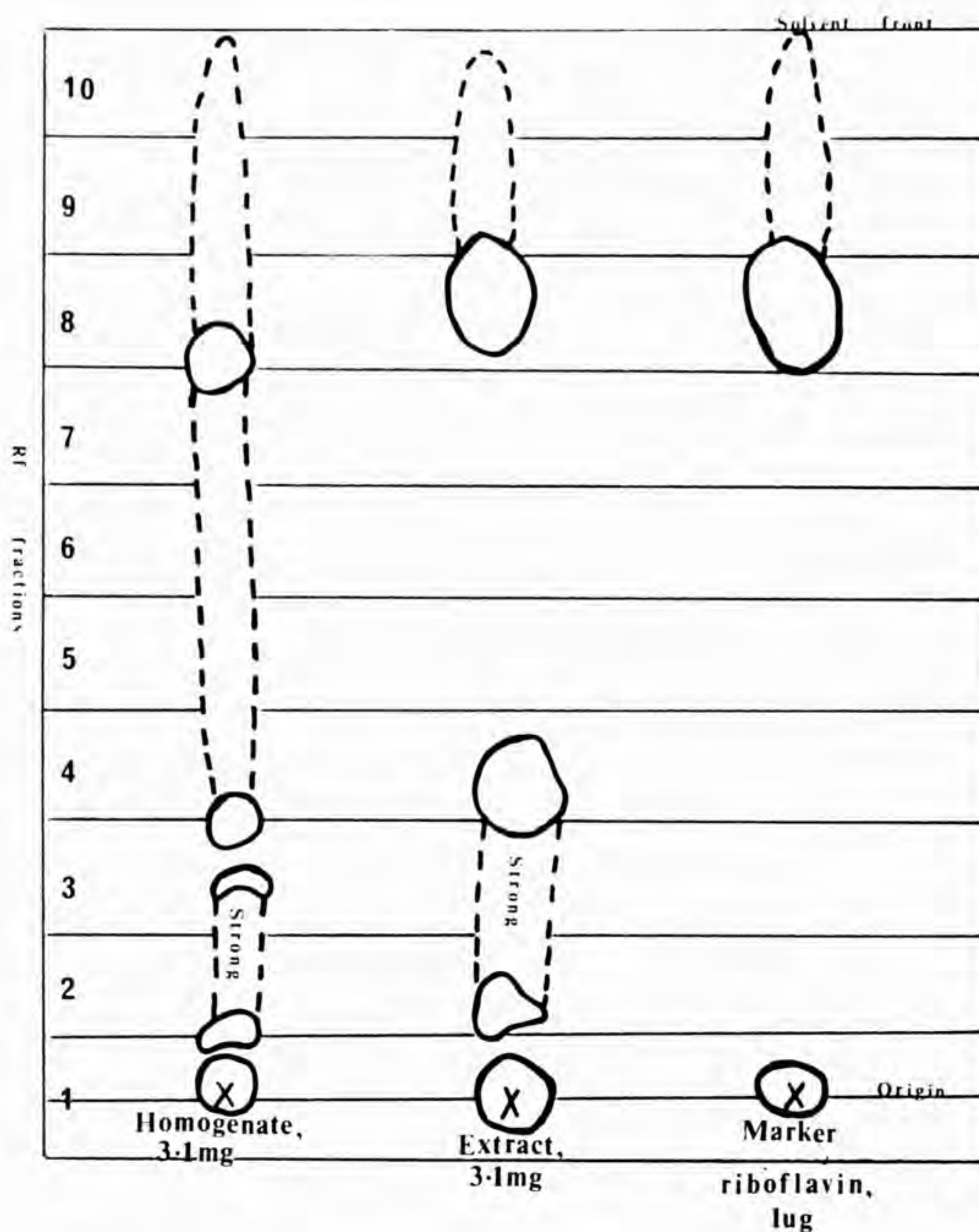


Fig. 62. Riboflavin present in excised tomato root extract and homogenate after fractionation in BuP.

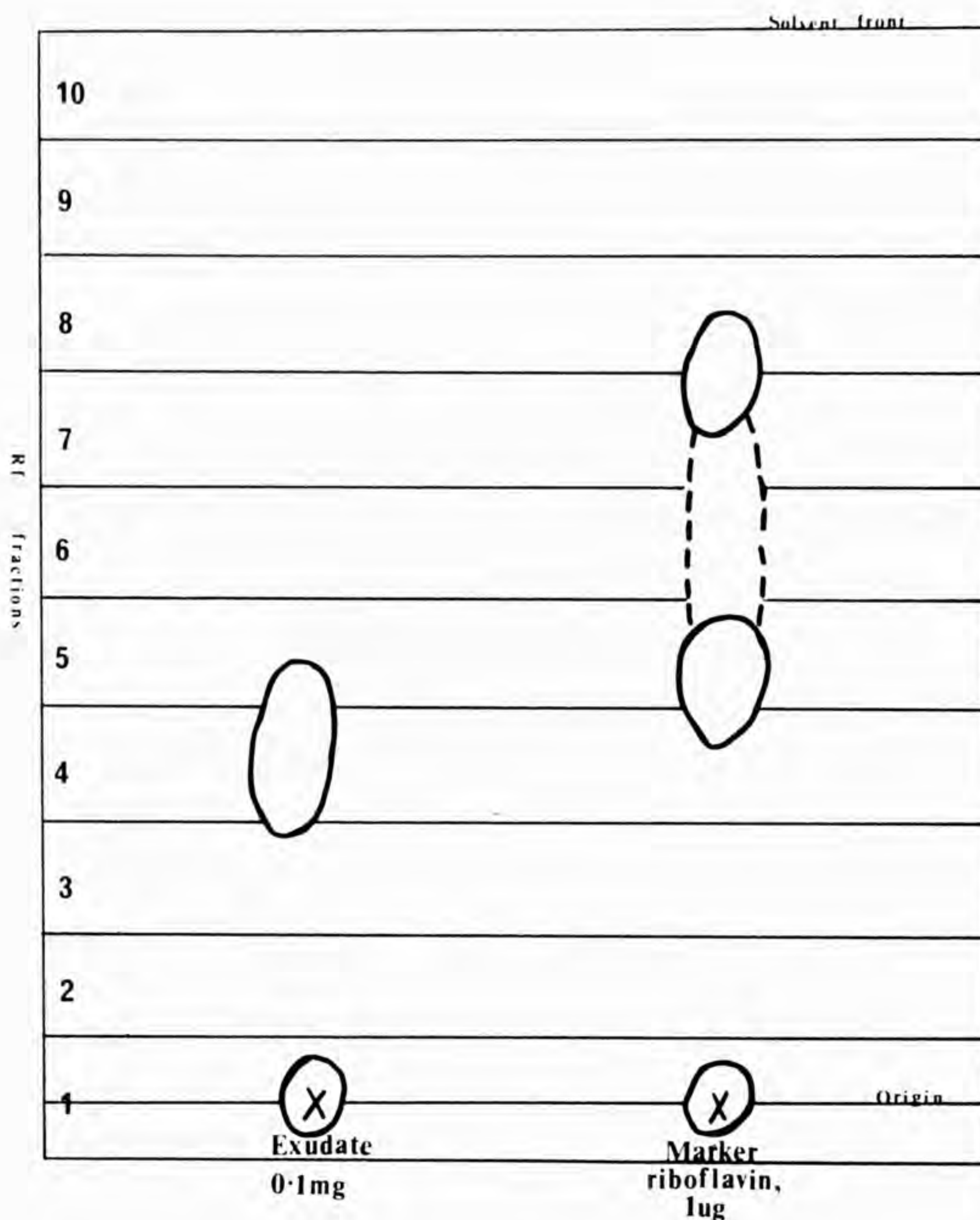


Fig. 63. Riboflavin present in pine seedling root exudate (collected for 3 weeks) after fractionation in BuA.

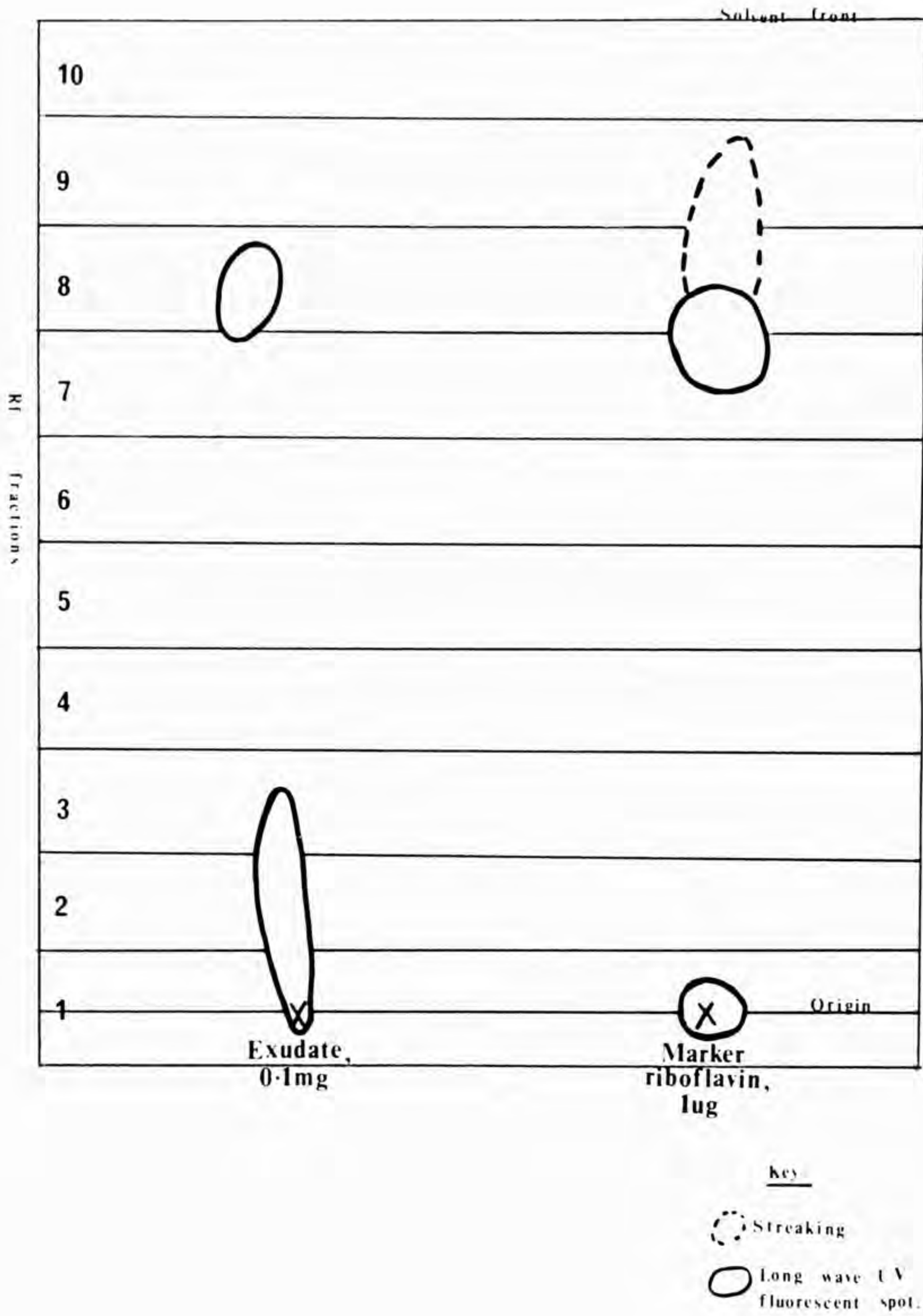


Fig. 64. Riboflavin present in pine seedling root exudate (collected for 3 weeks) after fractionation in BuP.

VITAMIN ASSAY.

1. Assays with *Escherichia coli* mutants.

The results of thiamin and niacin assays with excised tomato root exudate (from live roots, collected in distilled water), extract and homogenate using *E. coli* thi^- and *E. coli* nia^- are shown in Table 44.

Table 44.

Growth of *E. coli* thi⁻ and *E. coli* nia⁻ in the presence of live excised tomato root exudate, extract and homogenate.

Bacterium	Dilution of bacterium	Root preparation	Growth of bacterium (positive or negative)
E. coli thi$^-$	10^{-2}	No root preparation (control)	-
E. coli thi$^-$	10^{-2}	Thiamin	+
E. coli thi$^-$	10^{-2}	Extract	+
E. coli thi$^-$	10^{-2}	Homogenate	+
E. coli thi$^-$	10^{-4}	No root preparation (control)	-
E. coli thi$^-$	10^{-4}	Thiamin	+
E. coli thi$^-$	10^{-4}	Exudate	+
E. coli nia$^-$	10^{-2}	No root preparation (control)	-
E. coli nia$^-$	10^{-2}	Niacin	+
E. coli nia$^-$	10^{-2}	Extract	+
E. coli nia$^-$	10^{-2}	Homogenate	+
E. coli nia$^-$	10^{-4}	No root preparation (control)	-
E. coli nia$^-$	10^{-4}	Niacin	+
E. coli nia$^-$	10^{-4}	Exudate	+

These results indicate that both thiamin and niacin are present in live excised tomato root exudate, extract and homogenate.

The Rf fractions of these vitamins were then determined in BuA and BuP solvents (see Table 45 and Fig. 65).

Table 45.

Rf fractions for the vitamins thiamin and niacin fractionated in BuA and BuP, as determined by the growth of *E. coli* thi⁻ and *E. coli* nia⁻.

Bacterium	Rf fraction	Number of colonies of *E. coli* thi⁻ or the presence or absence of growth with *E. coli* nia⁻ in:-	
		BuA	BuP
E. coli thi⁻	No thiamin (control)	4	1
E. coli thi⁻	Thiamin	134	206
E. coli thi⁻	1	31	20
E. coli thi⁻	2	40	9
E. coli thi⁻	3	130	45
E. coli thi⁻	4	156	58
E. coli thi⁻	5	140	130
E. coli thi⁻	6	187	140
E. coli thi⁻	7	27	196
E. coli thi⁻	8	5	144
E. coli thi⁻	9	25	6
E. coli thi⁻	10	24	6
E. coli nia⁻	No niacin (control)	-	-
E. coli nia⁻	Niacin	+	+
E. coli nia⁻	1	-	-
E. coli nia⁻	2	-	-
E. coli nia⁻	3	-	-
E. coli nia⁻	4	-	-
E. coli nia⁻	5	-	Slightly +
E. coli nia⁻	6	-	+
E. coli nia⁻	7	-	-
E. coli nia⁻	8	+	-
E. coli nia⁻	9	+	-
E. coli nia⁻	10	-	-

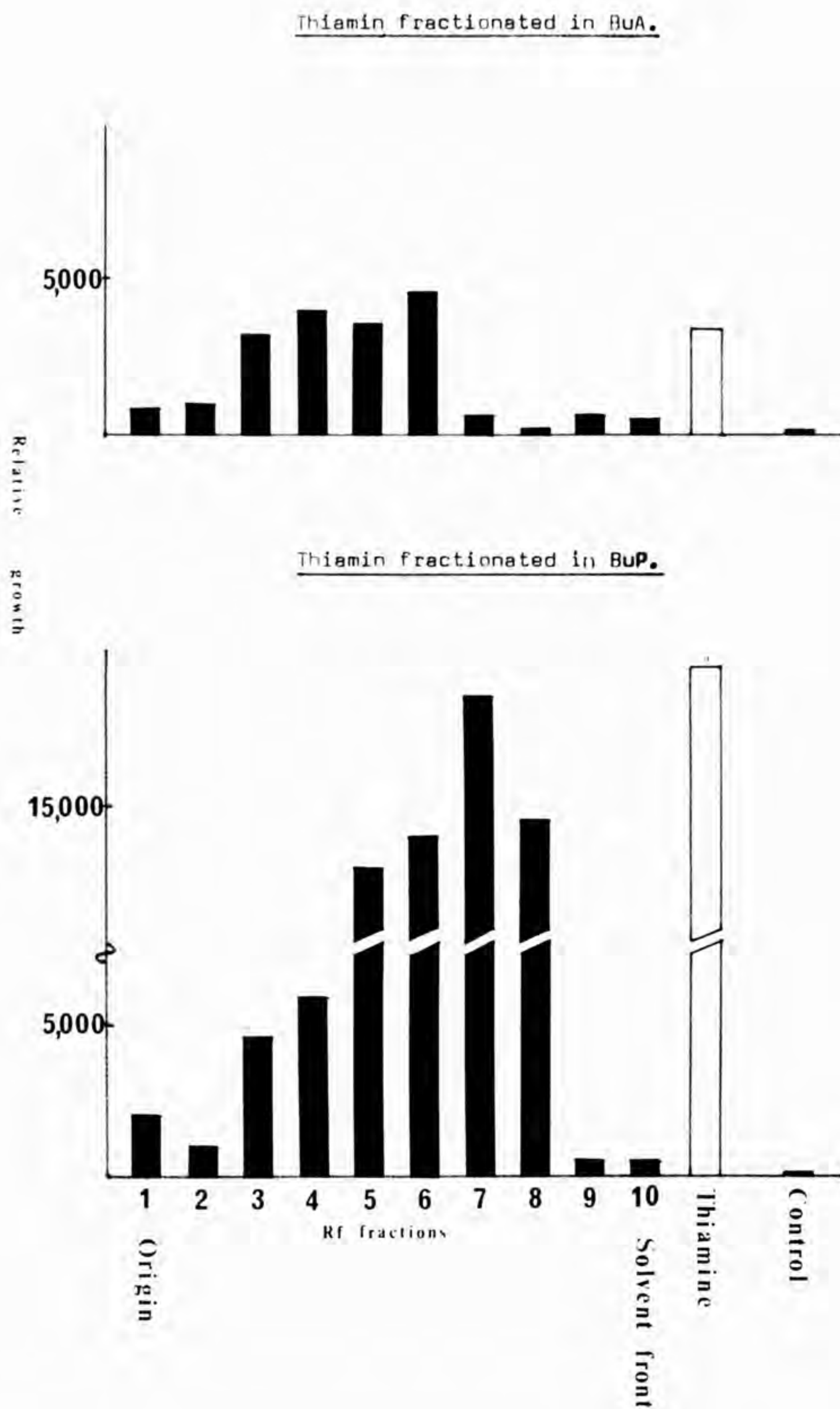


Fig. 65. Rf fractions for thiamin fractionated in BuA and BuP as determined by the growth of Escherichia coli thi⁻.

The Rf fraction for thiamin in BuA was 3–6 and in BuP 5–8 (Fig. 65), whereas the Rf fraction for niacin was 8–9 in BuA and 4–5 in BuP (Table 45). These values do not correspond closely with the unknown M13 growth stimulatory factor(s).

2. Assays with *Aspergillus nidulans* mutants.

The results of biotin and pyridoxine assays with excised tomato root exudate (from live roots, collected in distilled water), extract and homogenate, using *A. nidulans* bio⁻ and *A. nidulans* pyr⁻ are shown in Table 46 and Fig. 66.

Table 46.

Growth of *A. nidulans* bio$^-$ and *A. nidulans* pyr$^-$ in the presence of excised tomato root exudate (live), extract and homogenate.

Organism	Root preparation	Mean radius of colony (mm)	Standard error	P
A. nidulans bio$^-$	No root preparation (control)	0•6	(0•1)	-
A. nidulans bio$^-$	Exudate	5•7	(0•2)	0•001
A. nidulans bio$^-$	Extract	1•4	(0•2)	0•02
A. nidulans bio$^-$	Homogenate	1•5	(0•0)	0•001
A. nidulans bio$^-$	Biotin	3•0	(0•0)	0•001
A. nidulans pyr$^-$	No root preparation (control)	1•0	(0•0)	-
A. nidulans pyr$^-$	Exudate	2•9	(0•2)	0•001
A. nidulans pyr$^-$	Extract	2•4	(0•2)	0•001
A. nidulans pyr$^-$	Homogenate	4•4	(0•2)	0•001
A. nidulans pyr$^-$	Pyridoxine	5•9	(0•2)	0•001

Biotin and pyridoxine were both present in the live excised tomato root exudate, extract and homogenate. More biotin was present in the excised tomato root exudate (i.e. more than 1ug/ml agar) than in the excised tomato root extract or homogenate (i.e. less than 1 ug/ml agar). With the pyridoxine more was present in the excised tomato root homogenate, than in the extract or exudate of excised tomato roots and in all root preparations the amount of

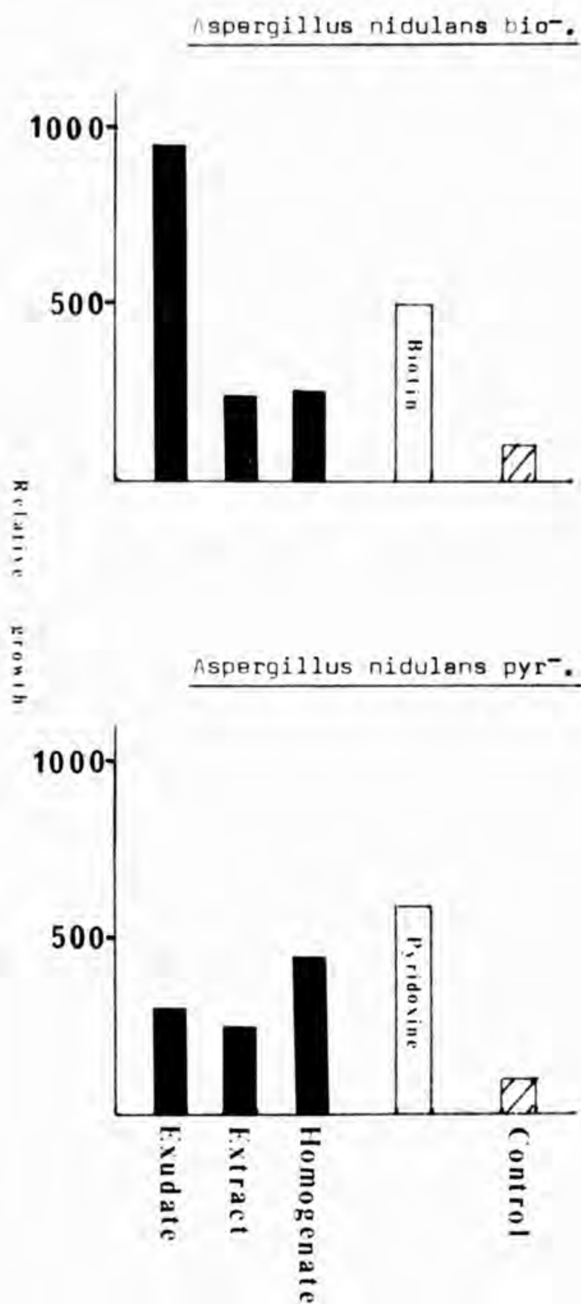


Fig. 66. Growth of Aspergillus nidulans bio⁻ and Aspergillus nidulans pyr⁻ in the presence of excised tomato root exudate (live), extract and homogenate.

pyridoxine was less than 1ug/ml.

The Rf fractions for biotin were then determined (see Table 47 and Fig. 67).

Table 47.

Rf fractions for biotin fractionated in BuA and BuP as determined by the growth of A. nidulans bio⁻.

Rf fraction	Radius of colony (mm) in:-	
	BuA	BuP
No biotin (control)	1•0	1•0
Biotin	3•5	3•5
1	3•5	3•0
2	1•5	1•0
3	1•5	0•5
4	1•5	3•5
5	1•5	3•5
6	3•0	3•0
7	4•5	3•5
8	4•0	1•0
9	2•5	0•5
10	1•0	0•5

From Fig. 67 the Rf fractions for biotin in BuA were 1 and 6-9 and in BuP 1 and 4-7. The Rf fractions for pyridoxine were determined by long wave UV light as being at Rf fraction 6-7 in BuA and 9 in BuP, although the UV light was not sensitive enough to determine the presence of

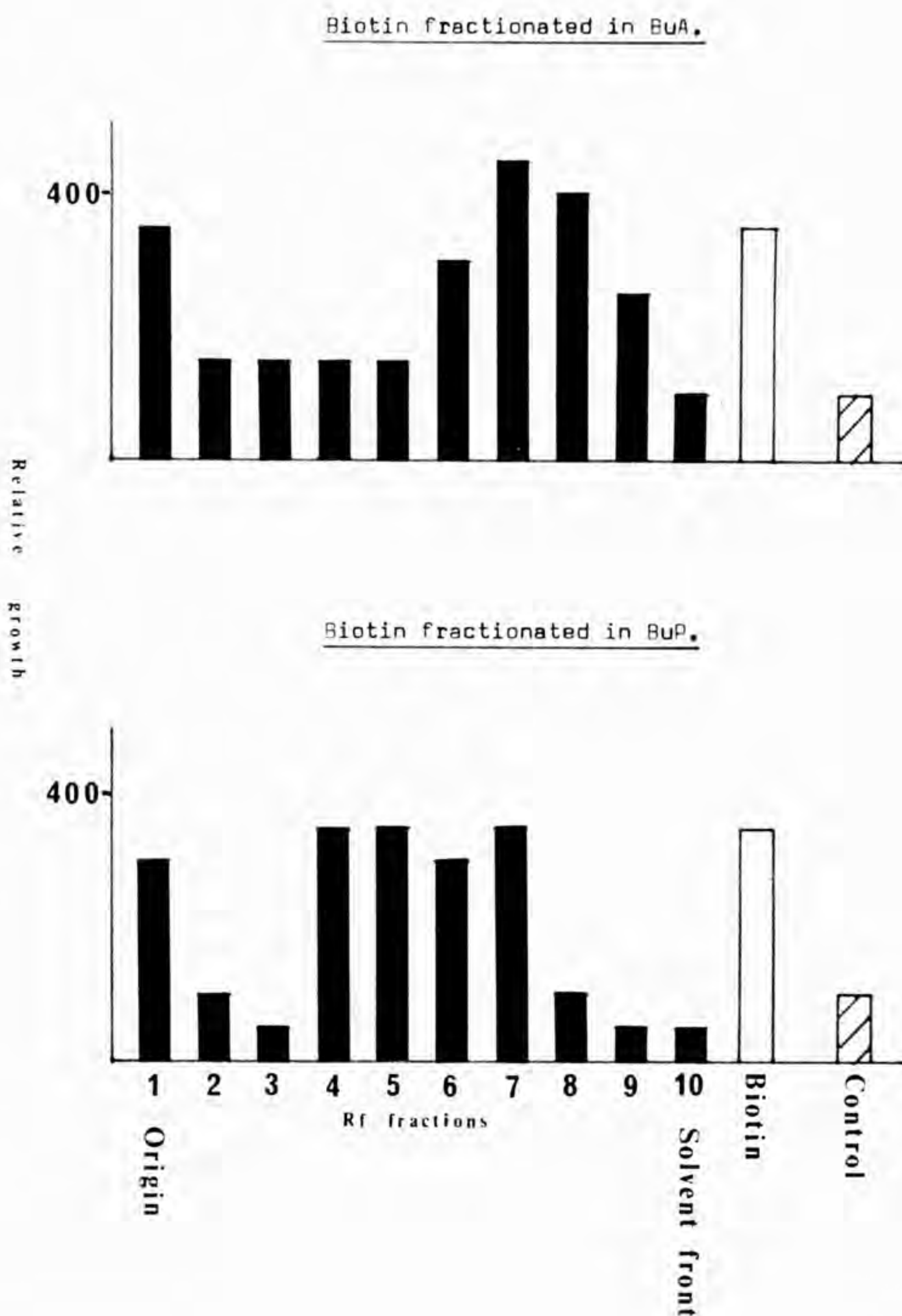


Fig. 67. Rf fractions for biotin fractionated in BuA and BuP as determined by the growth of Aspergillus nidulans bio⁻.

pyridoxine in either excised tomato root exudate, extract or homogenate.

Hence these two vitamins do not correspond to the M13 growth stimulatory Rf fractions of excised tomato root exudate, extract or homogenate and pine seedling root exudate.

IDENTIFICATION OF RIBOFLAVIN USING THE MASS SPECTROMETER.

The results obtained by this method of analysis seem to indicate that in fact riboflavin does correspond to the Rf fractions where growth promotion occurred (see Figs. 68 to 73).

The mass spectra of riboflavin and excised tomato root extract after their fractionation in BuP (Figs. 69 and 70) show a very similar pattern of peak formation, with the highest m/e (mass - to - charge ratio) value being at 223 (Rf fraction 1) and 278 (Rf fraction 7), hence these two Rf fractions have different maximum m/e values.

Similar mass spectra for riboflavin and excised tomato root extract were also obtained after fractionation in BuA (Figs. 71 and 72). There was no peak at m/e 278 in either the riboflavin or excised tomato root extract Rf fractions, also peak m/e 223 was absent from both riboflavin Rf fractions and peak m/e 206 was not present in riboflavin Rf fraction 0-4.

In both the riboflavin and the excised tomato root extract Rf fractions, peaks at m/e 206 and 223 (when present) were always of equal height, also peaks m/e 104 and 105 were always present.

On comparison of the mass spectrum of the non-fractionated riboflavin (Fig. 68) with the previous mass spectra the peaks at the lower m/e values were more intense, but towards the higher m/e values the peaks were very similar. Also peak m/e 278 was present. The actual molecular weight of riboflavin is 376, but no peaks occurred above m/e 278 in either the fractionated or non-fractionated riboflavin or the fractionated excised tomato root extract.

A mass spectrum of the tomato root extract, fractionated in BuP, at Rf fraction 4 was also obtained (Fig. 73). A similar spectrum was formed as the fractionated riboflavin with the highest m/e

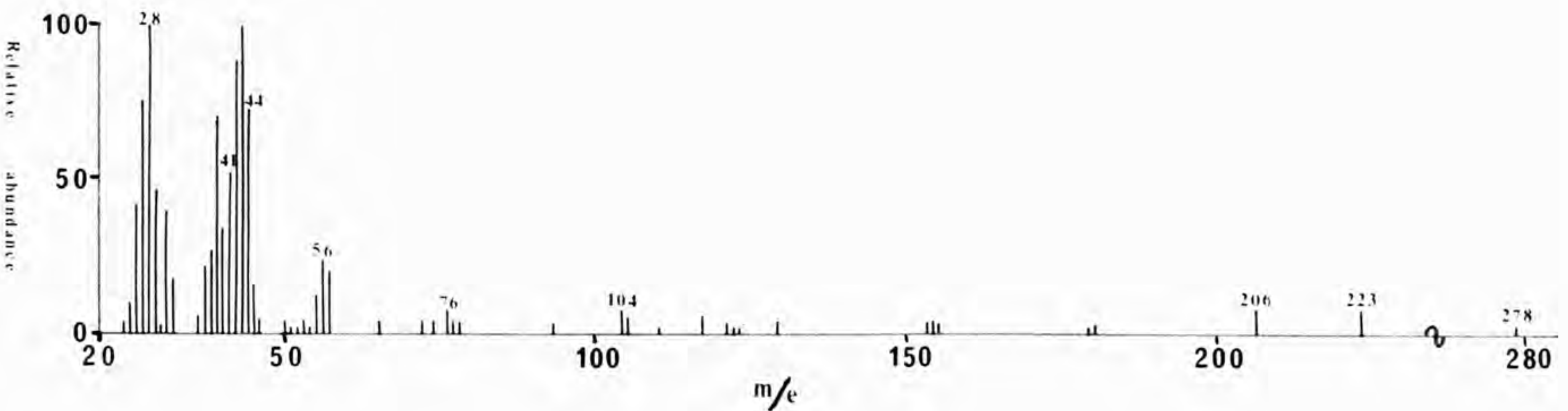


Fig. 68. Mass spectrum of non-fractionated riboflavin.

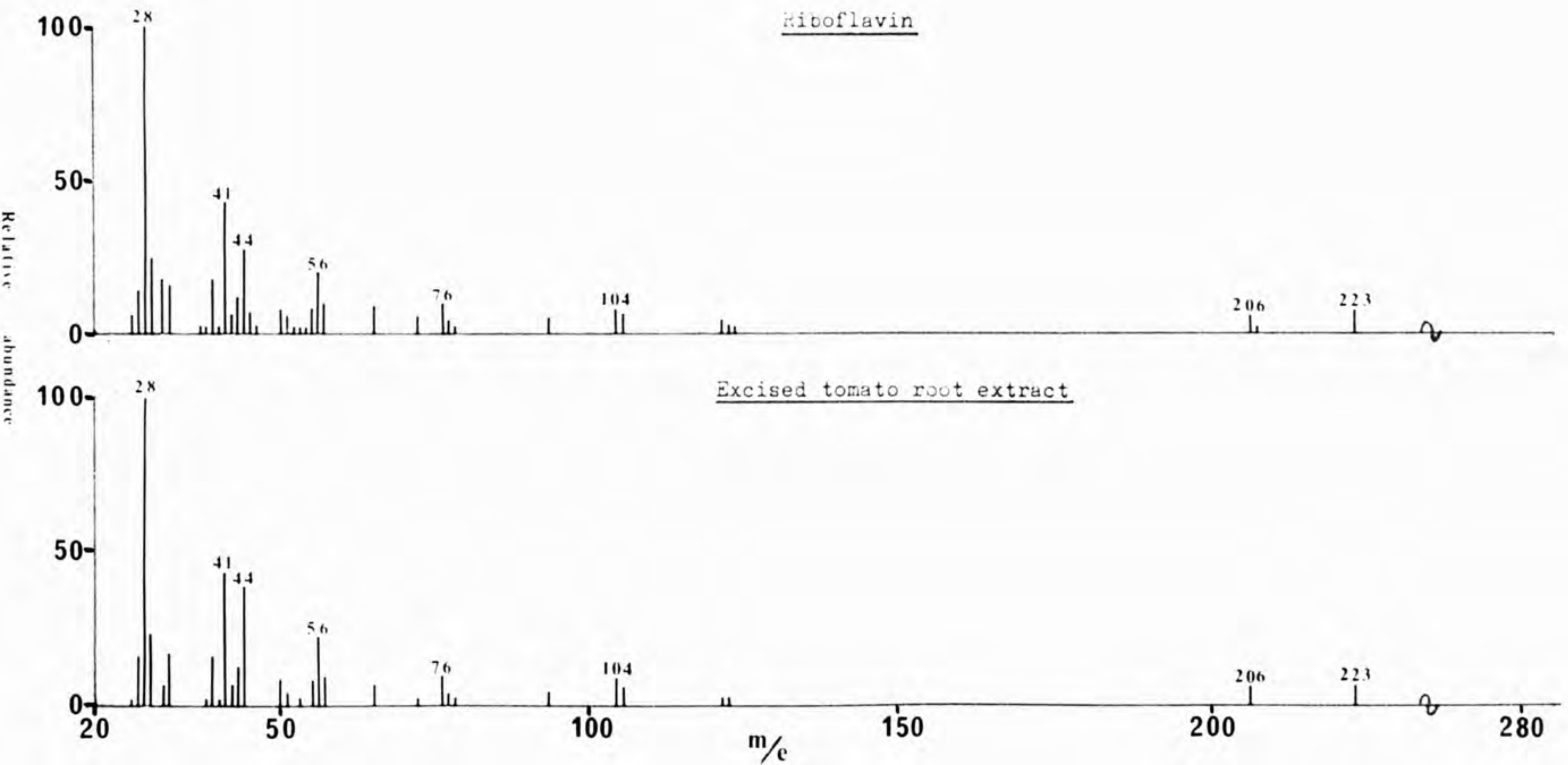


Fig. 69. Mass spectra of Rf fraction 1 from riboflavin and excised tomato root extract fractionated in BuP.

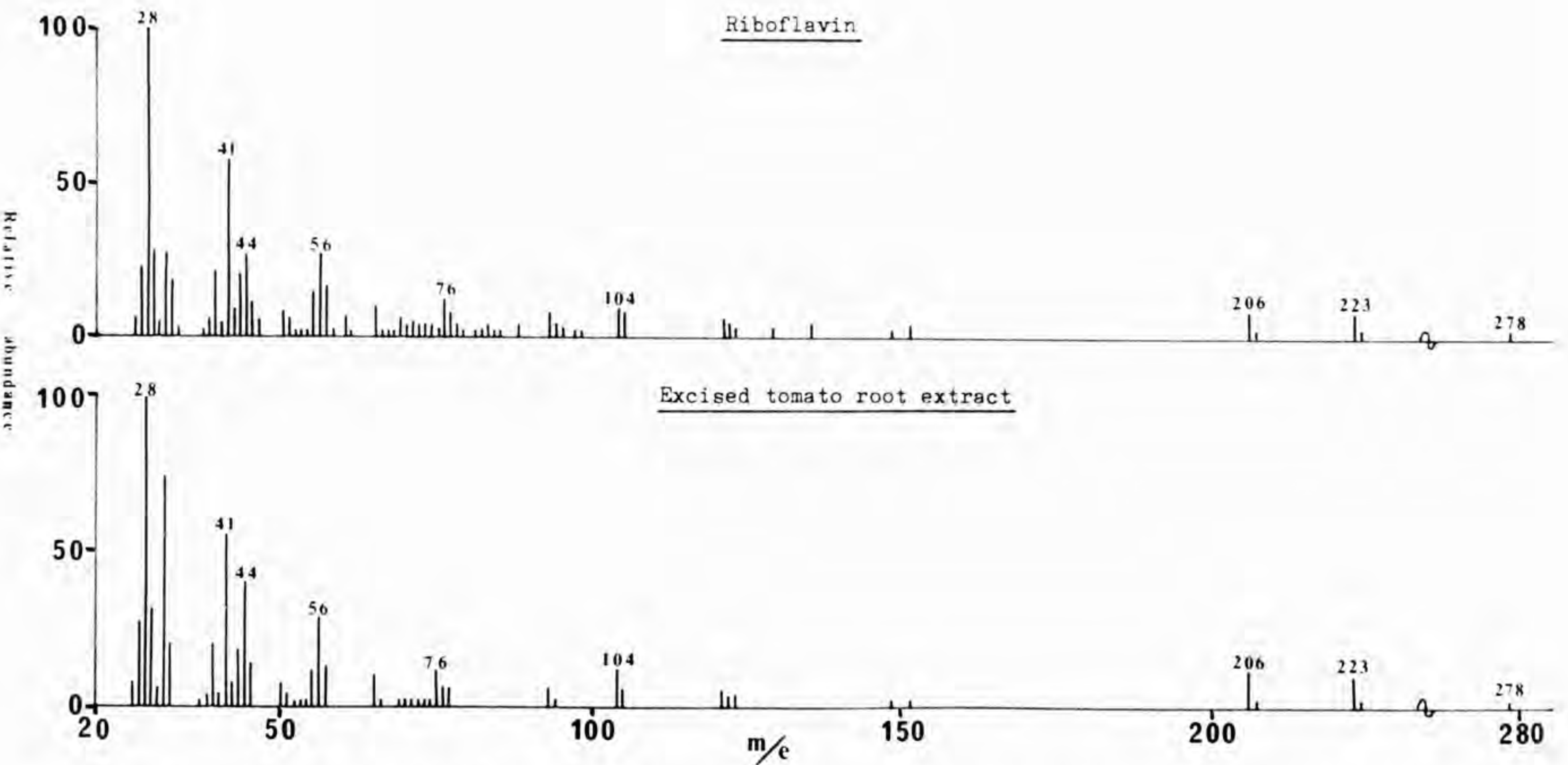


Fig. 70. Mass spectra of Rf fraction 7 from riboflavin and excised tomato root extract fractionated in BuP.

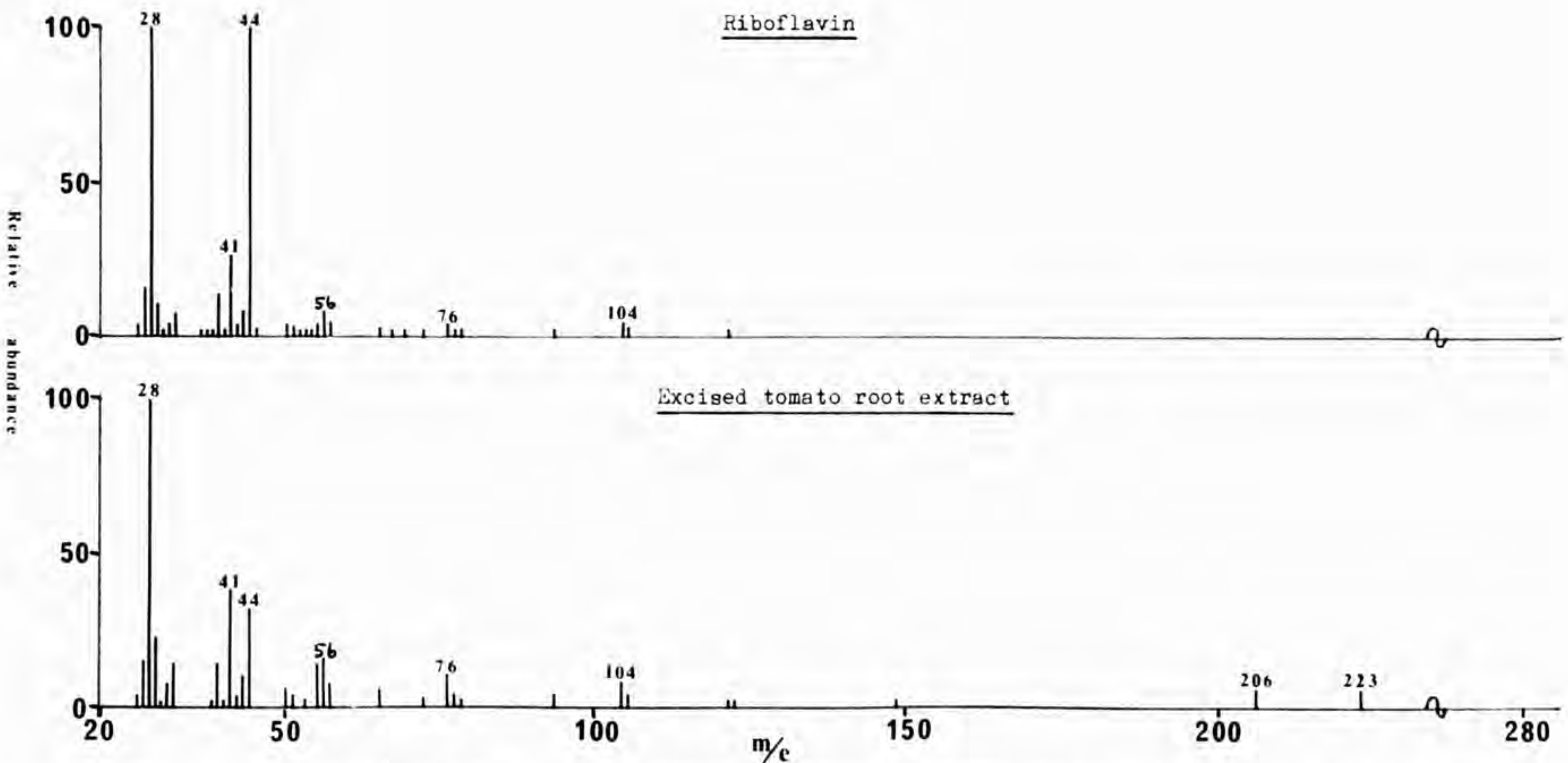


Fig. 71. Mass spectra of combined Rf fractions 0-4 from riboflavin and excised tomato root extract fractionated in BuA.

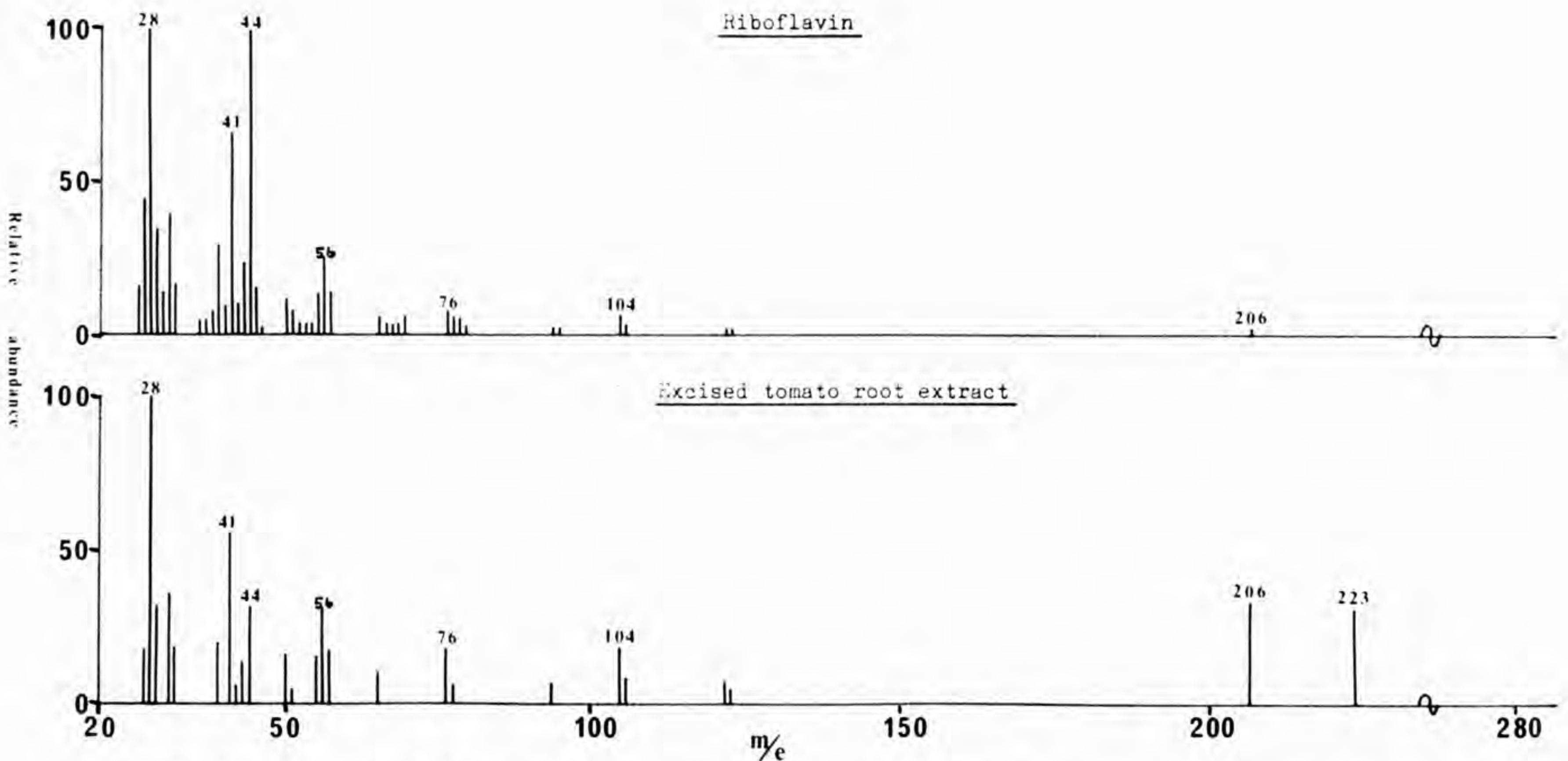


Fig. 72. Mass spectra of Rf fraction 8 from riboflavin and excised tomato root extract fractionated in BuA.

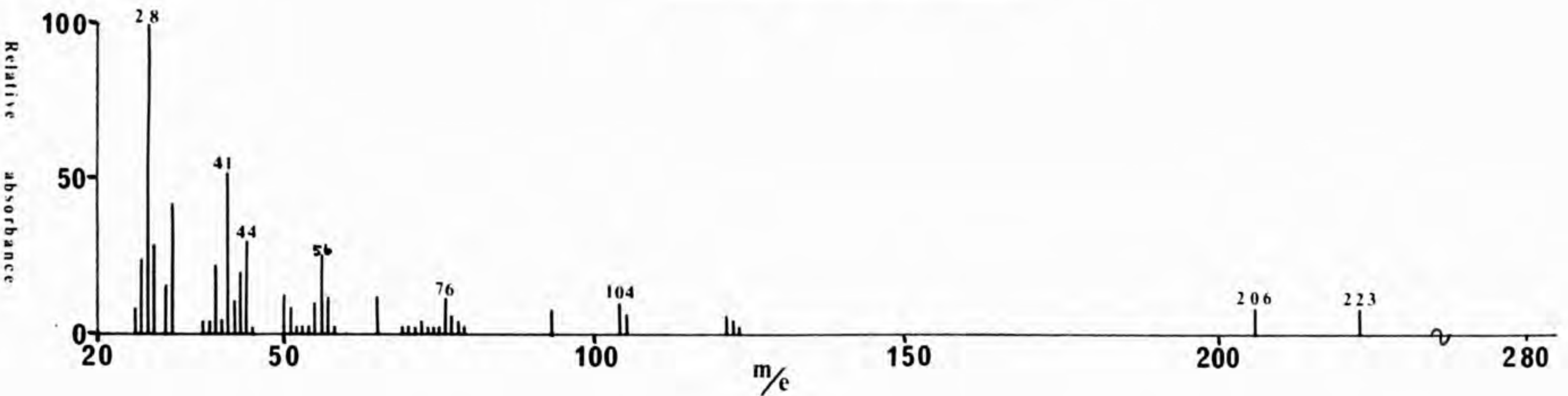


Fig. 73. Mass spectrum of Rf fraction 4 from excised tomato root extract fractionated in BuP.

being at 223, hence riboflavin was present. On examination of the chromatogram under long wave UV light it was noted that the riboflavin spots in this root preparation tended to streak across this Rf fraction. This would therefore account for its presence in Rf fraction 4, although it does not appear to be of a high enough concentration to produce M13 growth stimulation.

Therefore by the direct comparison of these spectra further evidence for the possibility that riboflavin could be the M13 growth stimulator(s) was obtained.

GROWTH EXPERIMENTS WITH COMPOUNDS IDENTIFIED IN EXCISED TOMATO ROOT PREPARATIONS.

1. Amino acids.

Table 48 and Fig. 74 show the effect of including individual amino acids in BLM (plus vitamins) on the growth of M13 and also the effect of supplementing MLM with these same amino acids.

Table 48.

The effect of individual amino acids on the growth of M13 in BLM (+ vitamin mixture) and MLM.

Amino acids	Dry weight in BLM plus vitamin mixture (mg)			Dry weight in MLM (mg)		
	Mean	Standard error	P	Mean	Standard error	P
No amino acids (control)	65•8	(7•7)	-	126•7	(13•5)	-
Isoleucine	81•8	(15•8)	n.s.	144•0	(13•6)	n.s.
Methionine	83•2	(6•5)	0•01	144•8	(11•9)	0•05
Valine	78•2	(14•2)	n.s.	130•3	(20•6)	n.s.
Alanine	99•1	(11•7)	0•05	147•0	(19•8)	n.s.
Lysine	101•1	(9•1)	0•02	141•1	(31•2)	n.s.
Histidine	92•4	(29•1)	n.s.	125•4	(1•2)	n.s.
Asparagine	87•6	(13•6)	0•02	143•7	(19•9)	n.s.
Serine	91•4	(9•6)	0•01	148•3	(12•8)	0•05
Aspartic acid	99•6	(23•2)	0•02	139•0	(31•9)	n.s.
Cysteine	76•3	(33•1)	n.s.	96•9	(14•2)	0•02*
No amino acids (control)	138•7	(20•3)	-	115•1	(15•6)	-
Tyrosine	123•5	(8•1)	n.s.	100•0	(13•4)	n.s.
Glutamine	139•2	(15•1)	n.s.	102•9	(10•3)	n.s.
Hydroxyproline	130•7	(15•0)	n.s.	95•5	(10•2)	0•05*
Mixture of above amino acids.	85•0	(9•0)	n.s.	101•3	(9•9)	n.s.

* Inhibition

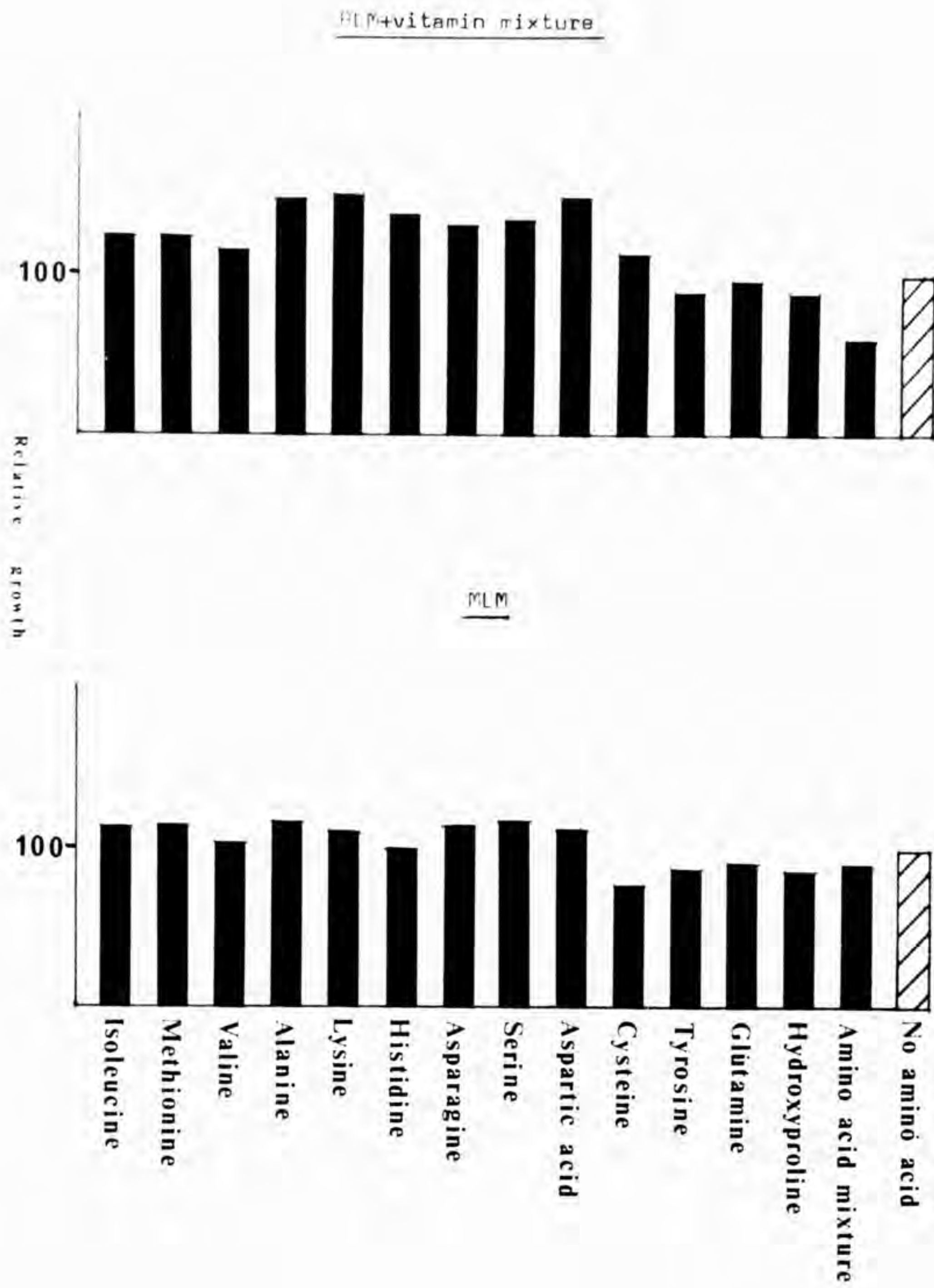


Fig. 74. The effect of individual amino acids on the growth of M13 in BLM (+vitamin mixture) and MLM.

A number of the amino acids stimulated the growth of M13 in BLM (plus vitamins), methionine and serine to the greatest extent, whereas some e.g. tyrosine and hydroxyproline had no significant effect on the growth of M13.

Similar results were obtained with individual amino acids in MLM, except that the stimulation of M13 was much reduced, and with cysteine and hydroxyproline significant inhibition of growth occurred. Hence these amino acids possibly do not correspond to the M13 growth stimulatory factor(s).

2. Sugars.

The growth of M13 in MLM in the presence of glucose, fructose or sucrose is shown in Table 49 and Fig. 75.

Table 49.

Growth of M13 in the presence of either glucose, fructose or sucrose in MLM.

Additional carbon source (0•05%)	Mean dry weight, mg	Standard error	P
No sugar (control)	117•2	(30•3)	-
Glucose	87•8	(12•1)	n.s.
Fructose	139•5	(26•0)	n.s.
Sucrose	95•9	(20•3)	n.s.
Sugar mixture (mixture of above sugars)	89•3	(4•9)	n.s.

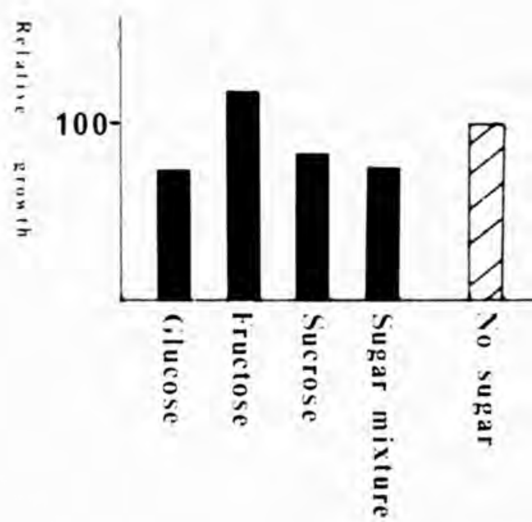


Fig. 75. Growth of M13 in the presence of either glucose, fructose or sucrose in MLM.

Glucose, fructose and sucrose, both individually and as a mixture had no significant effect on the growth of M13 in MLM. Therefore these sugars do not correspond to the M13 growth stimulatory factor(s).

3. Riboflavin.

The growth of M13 in MLM, in the presence of various concentrations of riboflavin is shown below in Table 50 and Fig. 76.

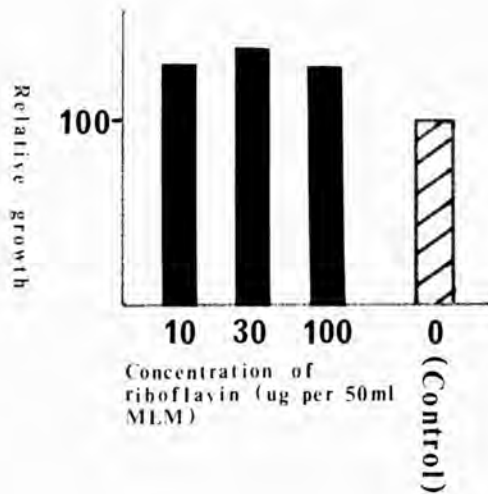
Table 50.

Growth of M13 in the presence of various concentrations of riboflavin in MLM.

Concentration of riboflavin (ug per 50ml MLM)	Mean dry weight (mg)	Standard error	P
0 (control)	70•2	(9•7)	-
10	94•1	(7•7)	0•01
30	100•4	(5•7)	0•002
100	93•2	(10•2)	0•02

All concentrations of riboflavin significantly stimulated the growth of M13, 30ug of riboflavin per 50ml of MLM giving the greatest growth stimulation. Hence this vitamin appears to be an M13 growth stimulatory factor.

Relative growth of M13 with riboflavin.



The effect of riboflavin on the growth of M13.

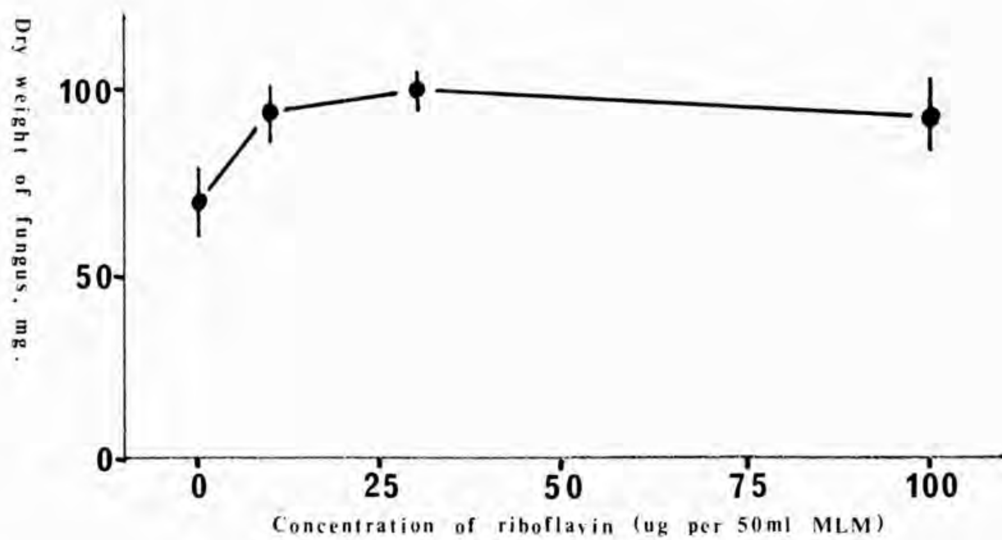


Fig. 76. Growth of M13 in the presence of various concentrations of riboflavin in MLM.

DISCUSSION

ISOLATION OF THE FUNGI FROM ECTOMYCORRHIZAL ROOTS OF PINUS SYLVESTRIS AND SPOROPHORES.

The isolation of fungi from the roots of surface sterilized ectomycorrhizae has been carried out by several workers (e.g. Zak and Marx, 1964; Lamb and Richards, 1970; Peña-Cabriales and Valdés Hgo, 1974).

Hydrogen peroxide (100 vols) was initially used in this investigation to sterilize the ectomycorrhizal roots for 30 minutes, this period of time producing the least number of contaminants. The hydrogen peroxide method was then replaced by sodium hypochlorite (1%), because the percentage number of fungi isolated from ectomycorrhizal roots sterilized with hydrogen peroxide was very low. Also the root isolates from hydrogen peroxide treated ectomycorrhizal roots did not form ectomycorrhizae *in vitro* with any of the ectomycorrhizal synthesis techniques used. It was speculated that perhaps the hydrogen peroxide had affected the ability of the root isolates to produce the host/fungus relationship, since an experiment using various concentrations of hydrogen peroxide showed that the growth rate of M13 over a period of 12 days was reduced by this substance.

The percentage number of fungi isolated after sterilizing the ectomycorrhizal roots with sodium hypochlorite was much higher, but again these root isolates were unable to produce ectomycorrhizae with the roots of *Pinus sylvestris* seedlings *in vitro*. Therefore any deleterious effect of hydrogen peroxide on the ectomycorrhizal forming ability of these isolates could not alone explain the lack of ectomycorrhizal formation. However the possibility of a selective action of these sterilants against isolating ectomycorrhizal-

forming fungi must be considered. It seems more likely that conditions condusive to ectomycorrhizal formation with these isolates may not have been used and this possibility is discussed in a later section (see page 288).

Root and sporophore isolates were maintained under mineral oil on agar slopes. Ferry (1967) noted that cultures not maintained under oil showed changes in their growth vigour, possibly by a process of inadvertent selection of sectors. The results of an experiment using the root isolate M13, maintained in a continuous laboratory culture, indicate that over a period of two years there was some loss in ability of this root isolate to respond to the presence of live excised tomato roots. However the slight loss in ability to respond to these roots did not seriously affect experimental results.

Two root isolates were specifically identified. M78 was identified as Verticillium psalliotae, this being an Ascomycete and a widespread pathogen of various Agarics, but can also be isolated from leaf litter and soil. There is no evidence that it is ectomycorrhizal, nevertheless, it should be noted that V. psalliotae was stimulated considerably by both killed and live excised tomato roots in MLM, although excised tomato roots do not appear to stimulate non-ectomycorrhizal Hymenomycetes (Ferry, 1967). M12 was identified as an Acremonium sp, these being ubiquitous saprophytes. No investigations were made as to the response of this isolate to excised tomato roots because it did not grow successfully in liquid culture.

In a few cases two distinct fungi were observed to grow from one ectomycorrhizal root, e.g. M59A and M59B. This confirms those observations made by Zak and Marx (1964) and suggests the possibility

that more than one fungus might be involved in the formation of a single ectomycorrhizal root.

Several of the root isolates obtained from different roots appeared to be morphologically similar when grown on BIA. This suggests that some isolates may have been a single widespread species, not necessarily confined to one area, e.g. the two morphologically similar isolates, M79 and M80, were isolated from ectomycorrhizal roots collected from Angley Woods and Brandon Forest respectively.

Three of the root isolates were black in colour and were very similar to, but not positively identified as Cenococcum graniforme.

All the root isolates grew into, as well as on the surface of the agar and were septate, but they all appeared to lack clamp connections. Clamp connections were also lacking in a number of unidentified ectomycorrhizal root isolates, isolated by Lamb and Richards (1970).

In order to determine whether these root isolates were Basidiomycetes or Ascomycetes, their ultrastructure was examined using the electron microscope.

The dolipore septa typical of Basidiomycetes and also occurring in the ectomycorrhizal fungi growing within the Hartig net and mantle (Foster and Marks, 1966), occurred in the septa of Boletus luteus, but were not observed in the septa of M1, M3 or M8. Hence either they were not present or they were not seen in the sections examined. In one root isolate, M1, a simple pore was present in one of the septa which indicates that this fungus could possibly be an Ascomycete. Although M1 seems not to be a Basidiomycete it could be an ectomycorrhizal-forming fungus, since a number of Ascomycetes, e.g. Tuber brumale (Fontana and Fasolo Bonfante, 1971) can form

ectomycorrhizae with Pinus nigra and Cenococcum graniforme can

form ectomycorrhizae with a number of different tree species.

ECTOMYCORRHIZAL SYNTHESIS AND THE ROOT MORPHOLOGY OF PINUS SYLVESTRIS SEEDLINGS.

The seeds of Pinus sylvestris used in these experiments were collected by the Forestry Commission from trees grown at Brandon Forest, Norfolk, in stands similar to those from which the ectomycorrhizal roots (and hence some of the root isolates) were collected. The reason for using this seed was to produce Pinus sylvestris seedlings which were hopefully compatible with the root isolates from this region.

Hydrogen peroxide, used as a method of seed sterilization, was very successful as indicated by Trappe (1961), with approximately 80% of the seeds germinating in 10 days.

The total lack of success using Fortin's method to synthesize ectomycorrhizae is not easily explained. Fortin (1966) obtained 40-42% of ectomycorrhizal root tips on Pinus sylvestris explants with Amanita rubescens at 21°C ± 1°C and using vermiculite as a substrate. Smith (1973) found that 32% of the roots inoculated with Thelephora terrestris and 38% of the roots inoculated with Amanita rubescens produced ectomycorrhizae using vermiculate as a substrate, but as stated by Smith the choice of media and the choice of fungal isolate is critical. It seems likely that, in these present experiments, conditions were not favourable for the production of ectomycorrhizae with either root isolates or Boletus luteus and Boletus variegatus1 rather than that the fungi were unable to form ectomycorrhizae. Both B. luteus and B. variegatus1 are proven ectomycorrhizal fungi (Trappe, 1962) and the former did form ectomycorrhizae under other conditions (see below).

Pachlewski's technique was also unsuccessful, although Pachlewski (1967) obtained 80% infection of Pinus sylvestris

seedling roots with Lactarius rufus and 100% with Rhizopogon roseolus, at room temperature in daylight. The inability of the root isolates and also B. luteus and B. variegatus1 to synthesize ectomycorrhizas with the pine roots grown in Pachlewski tubes was not due to the lack of fungal growth, for when the tubes were inoculated with a hyphal suspension the fungi were noted to grow extensively throughout the media. Again it must be assumed that the environmental conditions were probably not condusive to ectomycorrhizal formation.

To try to improve conditions pine seedlings were grown in a substrate similar to their natural environment, namely forest humus collected from areas where the ectomycorrhizal roots were obtained. Both sterile and non-sterile humus were used and seedlings were grown in pots, so that their shoots were exposed to the normal aerial environment. Again there was a marked lack of success, the only ectomycorrhiza formed being in a control (uninoculated) pot. Theodorou and Bowen (1970) using several isolates of Rhizopogon luteolus and Boletus granulatus obtained good ectomycorrhizal formation on Pinus radiata seedling roots in all inoculated plants, using soil which had been sterilized by gamma irradiation.

The technique used by Melin (1921, 1936) and later modified by Hacskaylo (1953) and Ferry (1967), where the pine seedlings were grown enclosed in flasks of perlite, was adapted by mixing the perlite with peat as recommended by Marx and Zak (1965) to maintain the pH at pH 4•6. Again no ectomycorrhizas formed, although the seedlings all appeared to be healthy. Marx and Zak (1965) obtained 4•6 ectomycorrhizas per centimetre of lateral root of Pinus elliottii with Laccaria laccata, 0•8 with Boletus luteus and 0•2 with Cenococcum graniforme at pH 4•6 using this method. However

the bases of the flasks were maintained in a constant temperature water bath and they were placed in a shaded greenhouse.

Since it appears advantageous to maintain the roots of the pine seedlings at a constant temperature and to have a controllable light source, an attempt was made to grow them in such a controlled environment. The roots of the pine seedlings (Pinus sylvestris) were maintained at a constant temperature of 20°C, and the shoots at a constant light intensity and photoperiod. This method was successful with Boletus luteus, ectomycorrhizae being produced on the roots of seedlings grown in both a dilute and normal mineral medium. The ectomycorrhizae, developed on pine roots in dilute medium were less abundant, and the shoots of these plants were chlorotic. Hence the concentrations of minerals in the substrate affect the degree of ectomycorrhizal formation as well as the ability of the seedlings to produce chlorophyll. There was no sign of ectomycorrhizal formation with any of the root isolates.

Although the in vitro synthesis of ectomycorrhizae with the root isolates failed, this is not conclusive evidence of an inability of these root isolates to form ectomycorrhizae as it is possible that these isolates could form ectomycorrhizae in the right environmental conditions.

Firstly it seems necessary to maintain the seedlings in a suitably controlled environment for the formation of ectomycorrhizae (as shown in this investigation with Boletus luteus), with an adequate light source and day length as suggested by Bjorkman (1942, 1944 and 1970) and the need to maintain the roots at a constant temperatue (Marx et al, 1970a). Also this method may need to be modified further by exposing the shoot to the atmosphere, since completely enclosing the shoot and hence the pine seedlings, exposes

it to an abnormally humid environment.

Pelham and Mason (1975) have shown different nutrient (phosphate) requirements for ectomycorrhizal formation with various strains of Betula verrucosa and Amanita muscaria. The root isolate, M13, does have different nutritional requirements to those of Boletus luteus (this is discussed in the next section, page 291) and hence it can be assumed that if this root isolate is ectomycorrhizal, the conditions for the formation of ectomycorrhizae would differ from those required by Boletus luteus.

Finally rhizosphere microorganisms are possibly an important factor in ectomycorrhizal formation in vivo. Certain bacteria present in the rhizosphere of the soil are known to be specifically present in the ectomycorrhizal roots of Pinus radiata infected with Boletus granulatus (Rambelli et al, 1972). Whether such rhizosphere microorganisms are essential to ectomycorrhizal formation is not known. It should be noted that endomycorrhizal infection by Endogone sp. could only be achieved in vitro in the presence of a certain species of Pseudomonas (Mosse, 1962). However when non-sterile humus was used in these ectomycorrhizal synthesis experiments, no ectomycorrhizas formed on the roots of Pinus sylvestris after inoculation with either the root isolates or the proven ectomycorrhizal fungus Boletus luteus. Hence the rhizosphere microorganisms present in the non-sterile humus were not conducive to ectomycorrhizal formation in these environmental conditions.

Although these root isolates did not produce ectomycorrhizae with the roots of Pinus sylvestris, they did, in some instances, influence their morphology. In explants of Pinus sylvestris, using Fortin's system for ectomycorrhizal synthesis with perlite as a substrate and normal medium, a few dichotomous non-ectomycorrhizal

secondary lateral roots were produced in the presence of M3 and Boletus luteus. Also non-ectomycorrhizal dichotomous secondary laterals were formed using Pachlewski's method for ectomycorrhizal synthesis on Pinus sylvestris seedlings in the presence of M1, M3, M7 and Boletus luteus. Morphologically these dichotomous roots were very similar to those produced during ectomycorrhizal formation, except that they showed no formation of Hartig net or mantle, although the hyphae did grow on and around the roots. Slankis (1949, 1950, 1951, 1958, 1963) noted that cell free culture filtrates from both Boletus luteus and Boletus variegatus produced dichotomies of pine roots similar to that obtained during ectomycorrhizal formation, and also that these dichotomies could also be produced by the exogenous application of indole-3-acetic acid (IAA). In this investigation, when IAA was applied at various concentrations to pine seedling roots grown on Pachlewski's starvation medium, only a very critical level of IAA (10^{-6}M), added four weeks after the seedlings had been transferred to the growth tubes, produced dichotomies. Pine seedling roots appear to be morphologically influenced by the presence of IAA, also these root isolates somehow supply an equivalent stimulus, which may or may not involve IAA itself.

NUTRITION OF THE ROOT ISOLATES.

The nutrition of the root isolates (mainly M13) was investigated in order to obtain more information concerning their requirements and to allow comparison with known ectomycorrhizal fungi.

M13 was able to utilize all the carbon sources tested well (except malate and acetate) and some (galactose, sucrose, mannose, mannitol and xylose) even better than glucose. These characteristics of M13 are similar to those of certain named ectomycorrhizal fungi. Palmer and Hacskaylo (1970) used six named ectomycorrhizal fungi including *Amanita rubescens* and found that mannose could be utilized more effectively than glucose with several of these isolates. Lamb (1974) noted that out of the 21 ectomycorrhizal fungi he tested, the Gasteromycetes, two species of *Thelephora*, *Xerocomus subtomentosus* and five unidentified fungi were capable of utilizing the largest number of carbon sources. Also the unidentified fungi which were shown to be important ectomycorrhizal forming fungi in some Australian pine plantations utilized many carbon sources (i.e. mannose, xylose, cellobiose, trehalose, glycogen and pectin) as well as or better than glucose. These fungi as well as *Boletus luteus* also adaptively grew on more carbon sources and utilized more in the absence of added glucose (0·1g C/l). He concluded that these named fungi mentioned above, which are dominant ectomycorrhizal forming fungi in forest nurseries, also the unnamed fungi (which are widespread in pine plantations) may be better adapted to survival in the soil, in the absence of a suitable host, than better known ectomycorrhizal fungi, since they are less fastidious in their carbon nutrition. However Melin (1925) found that *Boletus elegans* and *Boletus variegatus* grew well only in the presence of glucose,

other carbon sources being utilized to a lesser extent. This was confirmed by Ferry (1967) and Ferry and Das (1968) with various species of Boletus, including the two mentioned above.

All the polysaccharides tested were used well by root isolate M13, except CMC, which suggests that there is a lack of extracellular cellulase production in this root isolate. The ability of M13 to grow well in the presence of other polysaccharides conflict with those results obtained by Ferry (1967) with four species of Boletus where poor growth was recorded on all polysaccharides tested. However Lamb (1974) found that all the 21 ectomycorrhizal fungi (named and unnamed) grew well on both starch and pectin. This was confirmed by Palmer and Hacskaylo (1970) with pectin, although the pectin used in their investigation may have been impure.

Adaptive growth of ectomycorrhizal fungi with polysaccharides is known to occur in the presence of a trace amount of glucose (Norkrans, 1950; Ferry, 1967; Lamb, 1974) and a similar response occurred with M13 for some polysaccharides, especially with CMC. Hence M13 seems to adaptively produce extracellular cellulase(s). However pectin utilization was reduced in the presence of a trace of glucose.

Therefore, like those root isolates used by Lamb (1974), M13 is able to utilize a wide range of carbon sources including some polysaccharides. Hence it is also, perhaps, better adapted for saprophytic growth in forest soils, than many named ectomycorrhizal fungi, which are rather more fastidious in their carbon nutrition.

As mentioned previously M13 can utilize CMC only in the presence of a trace of glucose, but when this root isolate was grown on BIA containing macerated filter paper (i.e. cellulose) as a carbon source instead of glucose extensive but sparse growth occurred.

Similar results were obtained with root isolates M23, M37 and M65. Obviously growth recorded by this method is not comparable with mycelial growth on BIA (containing glucose) because of the different mycelial densities. Some comparison can be made, however, between these four root isolates without referring to control treatments containing glucose. All isolates appeared capable of utilizing cellulose to a significant extent. Growth of M13 in liquid medium containing CMC, where the actual dry weight of the fungus was recorded was probably more indicative of a limited ability of this fungus to utilize cellulose.

As cellulose does not usually occur free in nature, but physically combined with other plant material, the growth of the above four isolates from ectomycorrhizal roots i.e. M13, M23, M37 and M65 was determined on the sawdust of Pinus sylvestris wood. The ability of these root isolates to utilize the cellulose and lignin in this sawdust with and without a trace of glucose was observed. Only M13 significantly decomposed some of the cellulose in pine sawdust and, as with CMC, cellulose breakdown only occurred in the presence of a trace amount of glucose.

M13 was also able to significantly decompose the lignin, but again only in the presence of a trace of glucose, and hence there was apparent adaptive production of extracellular lignases by this fungus. M23 also seemed able to produce extracellular lignases, but only in the absence of glucose. Hence glucose suppressed lignin utilization by this root isolate. Two other root isolates (M37 and M65) were unable to decompose either the lignin or the cellulose in pinewood sawdust.

Some named ectomycorrhizal fungi are able to utilize lignin and/or cellulose. Norkrans (1949) found that, in the absence of

glucose, both the cellulose and lignin present in _Glyceria_ straw were utilized by three species of _Tricholoma_ (including _T. fumosum_) and that cellulose was decomposed in preference to lignin. Also the presence of a trace of glucose increased the lignin utilization of all species and caused adaptive growth of _T. personatum_ (as with M13).

Therefore the ability of M13 to utilize the cellulose and lignin in pinewood sawdust is no indication of not being an ectomycorrhizal fungus. In fact Norkrans (1950) suggested that some ectomycorrhizal fungi will remain in the ectomycorrhizal state as long as there is an excess of sugars in the host, but when these become limiting then cellulases are produced which break down cell walls to form ectendomycorrhizas, i.e. the fungi become more parasitic.

The optimum temperature for the growth of M13 was at 20°C, this being lower than the optima (25°C) of several species of _Boletus_ (Ferry, 1967), although _Rhizopogon roseolus_ has an optimum temperature of 13°C (Hacskaylo _et al._, 1965).

No optimum pH was obtained for M13, since it seemed to be below the range of the pH values used. However it should be noted that the natural habitat of this root isolate is in the very acid soil (humus) under pines, and its ability to withstand a very acid environment is, perhaps, another indication of its ability to survive in adverse conditions in the soil, which many named ectomycorrhizal fungi cannot tolerate so easily. Ferry (1967) reported the optimum pH of two ectomycorrhizal fungi, _Boletus bovinus_ and _Boletus variegatus_ as pH 5·0.

Many Hymenomycetes are known to prefer ammonium or an organic form of nitrogen for their growth. M13 utilized all nitrogen sources well at pH 4·6 showing little significant preference for either ammonium, peptone or nitrate. However it should be noted that the

ability of fungi to utilize certain nitrogen sources does depend on the pH of the medium. Other workers (e.g. Rawald, 1963) have noted that named ectomycorrhizal fungi e.g. Tricholoma vaccinum utilize nitrate poorly in comparison with ammonium as a nitrogen source.

M13, like most named ectomycorrhizal fungi, was completely heterotrophic for thiamin, this not being replaced by either the mixture of other vitamins, or the amino acid mixture, or a combination of these two.

In the presence of thiamin, amino acids stimulated M13 more than the vitamin mixture, but when the vitamins and amino acids were combined, a positive interaction occurred. A similar response was obtained with the four species of Boletus investigated by Ferry (1967). Of the other isolates tested for responses to vitamins (in addition to thiamin) and amino acids M1 showed increased growth in their combined presence, whereas M3 showed decreased growth.

In summary, there would appear to be no significant aspect of the nutrition of these present root isolates that precludes them from being ectomycorrhizal. There is evidence that some, e.g. M13, do use cellulose and lignin to a small extent but then so do some named ectomycorrhizal fungi. The ability of M13 to grow well at very low pH values and to utilize nitrate nitrogen efficiently may be of some significance.

ROOT EXPERIMENTS.

Ectomycorrhizal synthesis experiments, which were initially carried out during the course of this investigation, with several root isolates (including M13), suggest that these fungi are possibly not ectomycorrhizal forming fungi in vitro under those environmental conditions used in these experiments. However, as mentioned previously (see page 288), this is not conclusive evidence of an inability of these root isolates to form ectomycorrhizae in vivo.

As ectomycorrhizal fungi are known to be stimulated by both pine and tomato roots in pure culture, it was decided to investigate the effect of these roots on the growth of the root isolates, especially M13, and Boletus variegatus1 (a named ectomycorrhizal fungus). Also the responses of root isolate M13 and B. variegatus1 to various excised tomato root and pine seedling root preparations were compared. It was hoped that these results might, in the light of results obtained by other workers (Melin, 1954, 1962, 1963; Ferry, 1967) give information on the ecological status of M13.

The effect of roots and root preparations on the growth of M13 and B. variegatus1 are summarized below (see Table 51).

Table 51.

The effect of excised tomato and pine seedling root

Root preparation
1. Excised tomato root preparations.
Live root (7 days) in MLM.
Live root (14 days) in MLM.
Killed root (7 days) in MLM.
Killed root (14 days) in MLM.
Live root (14 days) in bag in MLM.
Killed root (14 days) in bag in MLM.
Cotton "root" (14 days) in MLM.
Cotton "root" (14 days)+live root exudate in MLM.
Extracted root (14 days) in MLM.
Extracted root (14 days)+live root exudate in MLM.
Live root exudate (14 days) in MLM.
Killed root exudate (14 days) in MLM.
Root extract (14 days) in MLM.
Root homogenate (14 days) in MLM.
½ Live root exudate (14 days) in MLM.
½ Killed root exudate (14 days) in MLM.
½ Root extract (14 days) in MLM.
½ Root homogenate (14 days) in MLM.
Live root exudate collected daily & supplied pooled (14 days) in MLM.
Killed root exudate collected daily & supplied pooled(14 days)in MLM.
Live root exudate collected daily & supplied daily (14 days) in MLM.
Killed root exudate collected daily & supplied daily(14 days)in MLM.
Live root exudate collected in MLM (14 days) in MLM.†

preparations on the growth* of M13 and Boletus variegatus1.

Method of sterilization and fungal isolate			
Micropore filtration		Autoclaving	
M13	B. variegatus1	M13	B. variegatus1
2•7	∞	-	-
4•3	2•7	-	-
1•8	1•0	-	-
2•1	0•5	-	-
0•7	-	-	-
0•9	-	-	-
1•5	-	-	-
1•6	-	-	-
1•8	-	-	-
1•7	-	-	-
1•4	0•7	1•4	0•8
1•4	0•4	2•2	0•4
1•9	1•0	1•7	0•4
2•0	5•2	1•9	0•7
0•9	-	-	-
1•3	-	-	-
1•4	-	-	-
1•6	-	-	-
1•0	-	1•6	-
1•0	-	1•3	-
0•7	-	1•3	-
1•2	-	1•2	-
3•3	-	3•9	-

Continued .....

Table 51. (cont.)

Root preparation
Live root exudate dialysate (14 days) in MLM.
Live root exudate retentate (14 days) in MLM.
Root extract dialysate (14 days) in MLM.
Root extract retentate (14 days) in MLM.
Root homogenate dialysate (14 days) in MLM.
Root homogenate retentate (14 days) in MLM.
Live root in MLM dialysate.
Live root in MLM retentate.
2. Pine seedling root preparations.
3 week exudate.
3 week extract.
3 week homogenate.
25 week exudate.
25 week extract.
25 week homogenate.

* a) Growth stimulation = $> 1\cdot0$

b) Growth inhibition = $< 1\cdot0$

† Root-depleted MLM.

Method of sterilization and fungal isolate			
Micropore filtration		Autoclaving	
M13	*B. variegatus*1	M13	*B. variegatus*1
1•2	-	1•2	-
1•3	-	1•2	-
1•4	-	1•7	-
1•4	-	1•7	-
1•8	-	1•5	-
1•7	-	1•7	-
0•8	-	0•3	-
1•4	-	0•0	-
0•8	0•9	1•0	1•4
0•9	1•4	1•3	1•6
1•1	1•2	1•0	1•2
4•4	<0•1	1•8	-
1•7	-	2•1	-
1•6	-	2•0	-

Boletus variegatus[1] was stimulated by live excised tomato roots in MLM, confirming the results obtained by other workers (Melin, 1962; Ferry, 1967). It did not respond to killed roots and again this is in general agreement with the results of other workers.

Melin (1962, 1963) in experiments with excised pine and excised tomato roots and with root exudates, concluded that *Boletus variegatus* was responding to both stimulatory and inhibitory components in the exudates. Net stimulation occurred at low exudate concentrations, and net inhibition at rather higher concentrations.

The critical concentration above which net inhibition of fungal growth occurred for excised pine roots was about 30 units per flask (Melin, 1963). One unit was defined as the exudate collected from 1mg dry weight of root over a 6 day period. Melin also noted that the critical concentration for pine seedling root exudate (from non-sterile grown plants) was much lower, about 8 units. For tomato roots there is no clear data in the literature, but Melin (personal communication to Ferry, 1967) states a critical concentration of $<$ 10 units per flask for excised tomato root exudate, and Ferry (1967) obtained increasing stimulation with up to 1·2 units per flask.

Melin noted that any treatment which disrupted root cell walls and membranes without completely disintegrating the roots e.g. freeze drying, increased the inhibition at any particular concentration of exudate, suggesting that the inhibitor(s) was not easily released from intact cells. The lack of stimulation of *Boletus variegatus*[1] with killed roots could well be due to a leakage of inhibitor(s) through damaged membranes rather than simply a lack of production of stimulator(s).

Melin also found that autoclaving root exudate resulted in net

increased stimulation and concluded from this that the inhibitor(s) was somewhat heat labile. Finally from other experiments involving roots extracted at 100°C, Melin (1963), concluded that a further non-diffusible stimulatory factor(s) was bound up within the root tissues. The present results need to be viewed in the light of these results of Melin with Boletus variegatus.

In the present experiments excised tomato root exudates, collected from live and killed roots used at a concentration of 6•2 units per flask inhibited the growth of Boletus variegatus 1. Root extract had no effect and root homogenate, the preparation of which involved maximum disruption of root cells, markedly stimulated Boletus variegatus 1. The inhibition obtained with root exudates must have involved leakage of inhibitor(s) from the cells, the effect being greater with killed root exudate derived from roots with damaged cell membranes. Thorough homogenization of the roots, however, resulted in a marked release of a stimulatory factor(s), presumably Melin's non-diffusible stimulatoryfactor. It would seem that the 6•2 units of excised tomato root exudate used in this experiment is above the critical concentration for stimulator v inhibitor of Boletus variegatus1.

At the same concentration, 6•2 units per flask, 3 week old pine seedling root preparations had no effect on Boletus variegatus 1 whereas 25 week old pine seedling root exudate was very inhibitory. This suggests a much greater quantity of inhibitor (or less stimulator) coming from the older roots. Melin (1963) has noted that older parts of pine root systems were much more inhibitory to Boletus variegatus than younger roots.

Little evidence was obtained to suggest that any root inhibitor(s) involved was heat labile. Autoclaving root preparations had little

effect on stimulation of Boletus variegatus 1. In fact, in the case of excised tomato root homogenate a massive stimulation obtained with micropore filtered preparation was converted to a significant inhibition.

Root isolate M13 was stimulated by both live and killed excised tomato roots. This fungus also exhibited growth stimulation in the presence of live and killed excised tomato root exudate and rather more stimulation with root extract and homogenate. The stimulation of M13 by exudate collected from killed roots, and by killed roots themselves suggests that it is less sensitive to any inhibitor(s) produced by roots compared with Boletus variegatus 1. Also like Boletus variegatus 1 no heat labile growth inhibitor(s) were detected in any of the root preparations, except in the exudate collected from killed excised tomato roots.

The difference in stimulation of M13 between roots and root exudates in PLM could be a purely physical effect, the fungus requiring some physical surface to grow on. The experiment with artificial cotton "roots" and also with leached extracted roots suggests that this may possibly be so. However a chemical explanation cannot be excluded because the roots do appear to contain cell bound stimulator(s). Also the cellulose (cotton) of the artificial roots and possibly of the extracted roots, may have been utilized to some extent by M13.

Like Boletus variegatus 1, M13 did not respond to 3 week old pine seedling root preparations, but it did show marked stimulation with all 25 week old pine seedling root preparations, especially live root exudate. Again this suggests that M13 is less subject to the effects of inhibitor(s) produced by roots than Boletus variegatus 1.

Further experiments in which exudate was collected daily from

live and killed excised tomato roots for up to 2 weeks added little information. No great stimulation or inhibition of M13 in MLM was obtained either when the exudate was added daily to the medium or as a single dose, added at the end of 2 weeks. However autoclaving these root preparations, did result in some stimulation of M13 growth.

In a final experiment, where exudate from live excised tomato roots was collected in MLM rather than water considerable growth stimulation of M13 occurred, comparable with that obtained with live roots and again this was enhanced when the exudate was autoclaved. There is therefore, some slight evidence for a heat-labile inhibitor affecting the growth of M13.

There is also evidence that roots exuding into MLM rather than distilled water might produce more diffusible stimulator(s). Melin (1962) found this to be true of pine seedling roots where he obtained greater stimulation of Boletus variegatus with exudate collected from seedlings grown at high nitrogen and phosphorus levels. However, another interpretation of the results of experiments involving exudate produced by live roots in MLM (and live roots in MLM), is that the living roots reduced the levels of certain nutrients, e.g. amino acids, in the MLM which were toxic to M13. Other workers (Melin, 1963; Ferry, 1967) considered that MLM might not be optimal for the growth of various ectomycorrhizal fungi and there is evidence in the present investigation that MLM is not entirely suitable for M13, e.g. with respect to carbon source and pH.

In summary, it can be said that like Boletus variegatus, M13 is stimulated by live tomato and pine roots. Non-mycorrhizal Hymenomycetes appear not to be stimulated (Lundeberg, 1960; Ferry, 1967) and this suggests that M13 might be ectomycorrhizal. However,

if both *Boletus variegatus* 1 and M13 are responding to the same factors in root exudates, then certainly they respond differently to varying concentrations of these factors. M13 seems generally to be less influenced by inhibitor(s) in root preparations than *Boletus variegatus* 1. Melin considers that such inhibitors may help to limit invasion of roots by fungi. Ectomycorrhizal fungi in particular seem to be limited to intercellular penetration of the pine root cortex and to the root surface only of tomato roots.

Other root isolates, tested with live and killed excised tomato roots in MLM, showed a range of responses. Many, like *Boletus variegatus* 1, showed stimulation with live roots, but not with killed roots. A few, like M13, responded slightly to killed roots and some were considerably stimulated by killed roots e.g. M3, M5, M22 and M23. It is possible that these latter isolates, and to some extent M13, are capable of a more saprophytic mode of life than "conventional" ectomycorrhizal fungi. It would be interesting to know whether species like *Tricholoma fumosum* and *Boletus subtomentosus*, known to be ectomycorrhizal and also capable of cellulose and lignin utilization (Norkrans, 1950; Lyr, 1963), respond to killed roots.

Experiments on the effect of root factors on M13 were continued further in an effort to isolate and identify at least the stimulatory factor(s) for M13.

Dialysis of live excised tomato root exudate, extract and homogenate gave surprising results. In all cases both dialysate and retentate produced stimulation of M13 growth, comparable to that obtained with non-dialysed root preparations.

It would appear that no separation of stimulatory and inhibitory components of these root preparations had occurred. However in further experiments in which live and killed excised

tomato roots enclosed in dialysis membrane bags were allowed to exude into MLM, some evidence was obtained for separation of root factor(s) into a somewhat dialysable inhibitory fraction and a less dialysable stimulatory fraction. However the separation does not seem to have been complete. There is no data available in the literature for the effect of excised tomato roots enclosed within dialysis membrane bags on the growth of ectomycorrhizal fungi. However Melin (1954) found that Boletus variegatus was stimulated on agar by pine roots enclosed within celluloid bags. This suggests that a Boletus variegatus stimulatory factor(s), exuded by the enclosed pine roots, was able to pass through the celluloid membrane.

Further experiments were then developed in an attempt to separate more successfully the stimulatory factor(s) into Rf fractions by paper chromatography. This method proved more successful than dialysis and specific M13 growth stimulatory Rf fractions were obtained in both BuA and BuP, with all excised tomato root preparations and pine seedling root exudates.

The amount of M13 growth stimulation obtained in the presence of the growth stimulatory Rf fractions was very similar to the non-fractionated root preparations, except pine seedling root exudate (collected for 25 weeks), when the massive growth stimulation which occurred in the non-fractionated root preparation was not evident after fractionation in BuA. Possibly, therefore, there was an interaction between all or specific Rf fractions in the non-fractionated pine seedling root exudate which significantly increased the growth stimulation of M13.

Fractionation of live excised tomato root preparations chromatographically also provided some further evidence for the existence of an M13 growth inhibitor(s). One such inhibitor(s)

occurred in the dialysate (from live exuding roots enclosed within dialysis membrane bags and collected in distilled water), fractionated in BuA and was produced after a growth stimulatory Rf fraction had been autoclaved, hence this stimulatory Rf fraction became toxic on autoclaving. A heat labile growth inhibitor was present in pine seedling root exudate (collected for 25 weeks), fractionated in BuA and also in both the extract and homogenate (at the same Rf fractions) of excised tomato roots, after fractionation in BuP, but not in BuA. However unlike the M13 growth stimulator(s), the inhibitory Rf fractions were not consistantly found in all root preparations, there being none present in live excised tomato root exudate or pine seedling root exudate, collected for 3 weeks.

All fractionated, like non-fractionated, root preparations of both excised tomato roots and pine seedling roots either inhibited or had no significant effect on the growth of Boletus variegatus 1. The Boletus variegatus 1 growth inhibitory Rf fractions in the excised tomato root preparations were both heat labile (e.g. extract fractionated in BuA) and heat stable (e.g. live root exudate fractionated in BuA). The Boletus variegatus 1 growth inhibitors in pine seedling root exudates were also heat labile, which thus confirms Melin's results (1963) with non-fractionated pine root exudate.

Therefore, to summarize, dialysing the excised tomato root preparations, as well as fractionating them chromatographically produced specific M13 growth stimulatory fractions. However, there is some evidence that an M13 growth inhibitor(s) does exist in excised tomato root preparations, since it was separated by dialysis from live and killed excised tomato roots, after they had been enclosed in dialysis membrane bags and from certain live

excised tomato root preparations and pine seedling root preparations (i.e. exudates) which had been fractionated chromatographically. Also as noted previously with non-fractionated root preparations, all Rf fractions were inhibitory to _Boletus variegatus_ 1 at concentrations which were stimulatory to M13.

Attempts to identify the M13 growth stimulator(s) led to the identification of many groups of substances present in live excised tomato root preparations.

Many amino acids were present in all the live excised tomato root preparations. Certain amino acids are known to stimulate the growth of ectomycorrhizal fungi _in vitro_ e.g. _Lactarius deliciosus_ is stimulated by a mixture of 18 amino acids similar to those found in casein hydrolysate, when grown in a nutrient solution containing glucose, mineral salts, ammonium tartrate and thiamin (Melin and Norkrans, 1948). M13 also showed a similar stimulation when an amino acid mixture was added to BLM. However MLM (which contains this amino acid mixture) was used as a growth medium in all these root experiments. Also when growth experiments were carried out, adding the amino acids identified in the excised tomato root preparations to MLM, both individually and as a mixture, there was no further significant M13 growth stimulation by any of the amino acids present within the M13 growth stimulatory Rf fractions. Therefore none of these amino acids replaced the M13 growth stimulatory factor(s).

A wide range of carbon sources are known to be utilized by ectomycorrhizal fungi grown in pure culture. Glucose is known to be the best source of carbon for many of these fungi (Melin, 1925; How, 1940; Norkrans, 1950; Ferry, 1967), and so in all of these root experiments the carbon source used in the growth

medium was glucose. However as noted previously (see page 291) this carbon source does not give optimal growth of M13, but the addition of one, or a mixture, of the carbon sources, present in the excised tomato root preparations, to the growth medium, did not significantly stimulate the growth of M13. As stated by Harley (1969), they would be a very non-specific growth stimulator.

Organic acids and also Ehrlich positive substances did not absolutely correspond to the M13 growth stimulatory fractions. Also several phenols and phenolic acids were detected, but they were not identified any further, for as suggested by Ferry (1967) they would be very unlikely to stimulate mycelial growth, although it could be that the M13 growth inhibitor(s) is a phenolic compound (s).

Vitamins are known to stimulate the growth of a number of ectomycorrhizal fungi. Thiamin is required either partially or totally by most ectomycorrhizal fungi (Chudjakow and Woznjakowskaja, 1951; Melin, 1953; Rawald, 1962). Also partial requirements for inositol by Rhizopogon roseolus (Melin and Lindeberg, 1939), pantothenic acid by Tricholoma imbricatum (Norkrans, 1950) and niacin by Lactarius deliciosus (Melin, 1953) are known to occur. Several vitamins, i.e. thiamin, niacin, biotin, pyridoxine and riboflavin were identified in all the excised tomato root preparations and one, i.e. riboflavin, which was also found in pine seedling root exudate collected for 3 weeks, had a similar fractionation pattern as the M13 growth stimulator(s). The riboflavin molecule on fractionation split into two parts, and it is these two Rf fractions which corresponded to the M13 growth stimulatory Rf fractions. This was also confirmed by the mass spectra of the fractionated excised tomato root extract.

There are no reports of any ectomycorrhizal fungi requiring

riboflavin in the literature. Nevertheless M13 growth experiments using various concentrations of riboflavin added to MLM suggest that M13 has a partial requirement for this vitamin, maximum growth stimulation occurring at a concentration of about 30ug per 50ml MLM. A similar amount of growth stimulation was also produced by M13 in the presence of both live and killed micropore filtered excised tomato root exudate. Hence it is probable that it is the riboflavin present in the excised tomato root exudate which is responsible for the growth stimulation of M13 in the presence of these root preparations, although further experiments need to be undertaken to determine the actual concentration of riboflavin in these excised tomato root exudates to see whether it is comparable with that added additionally to MLM i.e. 30ug of riboflavin per 50ml MLM. However the growth of M13 in the presence of both excised tomato root extract and homogenate, also with excised tomato roots themselves, was much greater than with either the micropore filtered live or killed excised tomato root exudate or riboflavin. Hence it can be assumed that since the concentration of riboflavin which produces optimal growth does not appear to be much above the value obtained with 30ug of riboflavin per 50ml MLM, there must be some other factor(s) involved. This also seems to apply to the pine seedling root preparations prepared from roots which had been exuding for 25 weeks into distilled water.

Melin (1963) did suggest that the M-factor does consist of two components, one being bound within the root cells and the other being able to pass through the root cell walls. Experimental results with these excised tomato root preparations support this possibility, since disruption of the cell walls either by extraction or homogenization both gave an increase in growth stimulation, possibly, therefore, due to the release of this cell bound M13 growth

stimulator(s). However neither root preparations stimulated the growth of M13 to such an extent as the excised tomato root itself, although as mentioned previously, the root was exuding into MLM. Hence it is quite probable that the root may alter some component of the MLM which thus causes a much greater M13 growth stimulation. This possibility was supported by the results with excised tomato root exudate collected in MLM and used without evaporation.

As mentioned previously pine seedling root preparations from roots which had been exuding for 25 weeks, also produced much more massive M13 growth stimulation than could be accounted for by riboflavin alone. However no attempt was made (due to lack of material) to determine whether riboflavin was present in these root preparations. Pine seedling root preparations, from roots which had been exuding for 3 weeks did not stimulate the growth of M13, although riboflavin was identified in the pine seedling root exudate. The quantity of riboflavin present in this pine seedling root exudate was much greater (approximately 31 times) than that found in a similar quantity (of equivalent dry weight of root) of excised tomato root. preparations, hence it is probable that this concentration of riboflavin was too high to cause M13 growth stimulation. Thus this may be the or one of the reasons why the pine seedling root exudate, collected for 3 weeks, and possibly the extract and homogenate from these roots, did not stimulate the growth of M13.

Another experiment besides this riboflavin growth experiment has also demonstrated the partial dependence of M13 on this vitamin. Growth of M13 in MLM which lacks the normal vitamin mixture, but which contains riboflavin at a concentration of 50ug per 50ml of medium, is similar to the growth which occurs in the presence of MLM (which includes the vitamin mixture). Hence the other vitamins

in this mixture appear to be of little additional value in the growth of M13 in MLM. It would also be interesting to see whether other ectomycorrhizal fungi (e.g. Boletus variegatus), respond to riboflavin and if so whether at the same or at a different concentration to that required by M13.

Therefore to summarize in finality, attempts to synthesize ectomycorrhizas on the roots of Pinus sylvestris in vitro with several of the root isolates were unsuccessful under those conditions used in these experiments. However these root isolates, especially M13, do have similar nutritional requirements to many named and unnamed ectomycorrhizal fungi isolated from sporophores and from ectomycorrhizal roots of conifers by e.g. Lamb (1974), although they do differ from some named ectomycorrhizal fungi e.g. Boletus variegatus (Ferry, 1967).

The results of root experiments show that like many ectomycorrhizal fungi, a large number of the root isolates are stimulated by live excised tomato roots and also one of these root isolates i.e. M13 is stimulated by both live excised tomato root preparations and pine seedling root preparations, which is in accordance with the results of Melin (1954, 1962 and 1963) using Boletus variegatus.

Nevertheless the concentration of root preparation which stimulated M13 was higher than that required to stimulate Boletus variegatus.

Attempts to identify this M13 growth stimulatory factor(s) led

to the identification of riboflavin as an M13 growth stimulator. However this vitamin is not the only factor involved, since the results of growth experiments with various concentrations of riboflavin have shown that maximum growth stimulation of M13 is not as massive as the excised tomato root itself.

Hence M13 growth stimulation is probably the result of a complex system of interacting growth factors which experimental evidence has shown to consist possibly of both cell bound and cell free growth stimulator(s) (including riboflavin), with M13 growth inhibitor(s) also being present.

Therefore to conclude, it seems that these root isolates, especially M13, do have many characteristics of named ectomycorrhizal fungi, responding to both excised tomato and pine seedling roots, also their nutritional requirements are similar to certain named and unnamed ectomycorrhizal fungi. Hence this evidence suggests that these root isolates are probably involved in ectomycorrhizal formation *in vivo*. However to confirm this, further experiments with these root isolates need to be undertaken to determine the conditions necessary for ectomycorrhizal formation *in vitro* (using the knowledge obtained from this investigation), and hence so to prove by ectomycorrhizal synthesis experiments, that these root isolates are ectomycorrhizal fungi.

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APPENDIX A

Mycelial morphology on BIA of two sporophore isolates and a selection of unnamed root isolates isolated from the ectomycorrhizal roots of *Pinus sylvestris*.

1. Colony characteristics of sporophore isolates. (See Fig. 77)

Boletus luteus.

Dark brown hyphae adpressed to surface of agar. White aerial hyphae sparse at perifery, but dense at the centre.

Undersurface:- Light brown margin to dark brown at the centre.

Boletus variegatus 1.

Dark brown with whitish-brown sparse aerial hyphae. Margin very irregular.

Undersurface:- Brownish-white margin to dark brown at the centre.

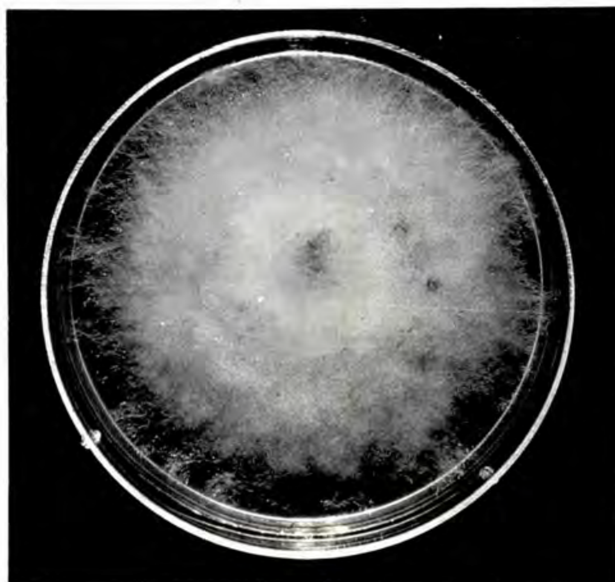
2. Colony characteristics of root isolates. (See Fig. 78)

M1.

Pure white to green to greenish-white at the centre due to the presence of coarse aerial hyphae. Hyphae at edge adpressed to and penetrating agar surface.

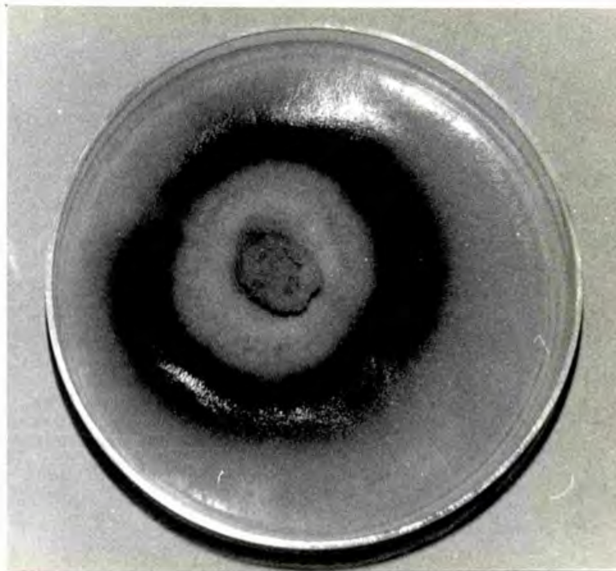
Undersurface:- White margin to green to cream at the centre.

Boletus luteus

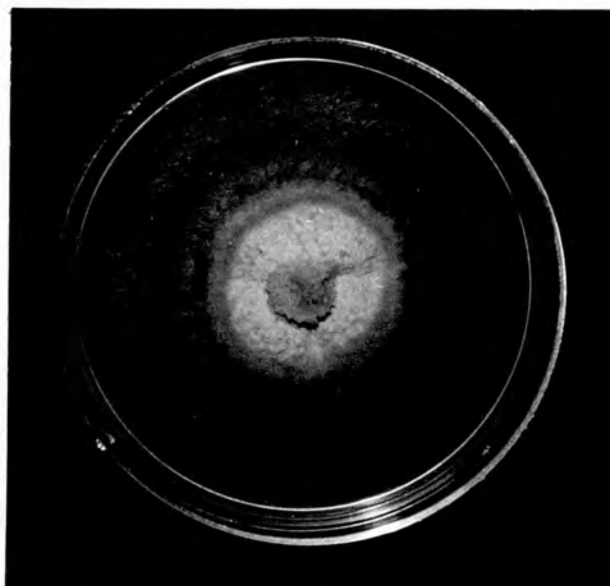


Boletus variegatus 1

Fig. 77. Sporophore isolates on BIA.



M1

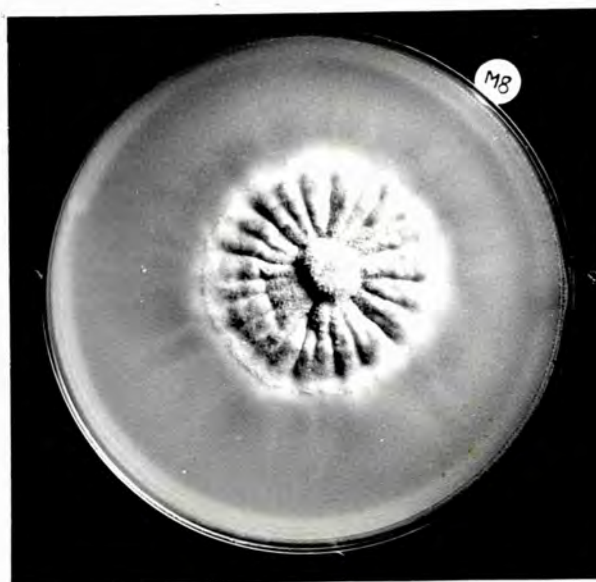


M3

Fig. 78. Root isolates on BIA.

Continued .....

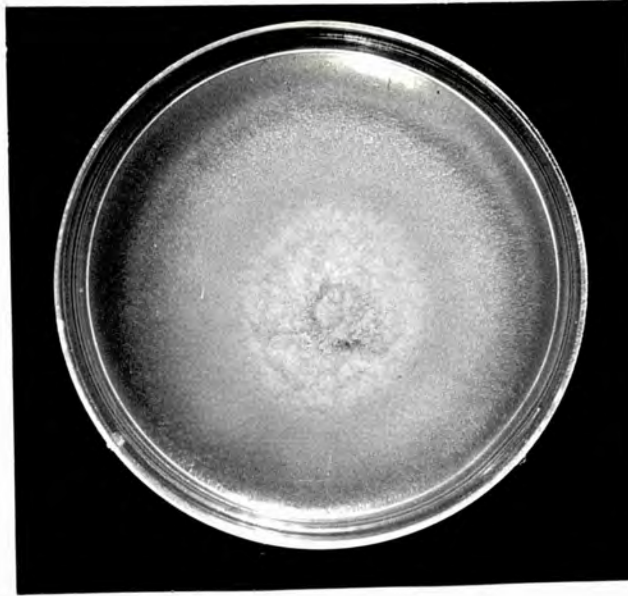
M7



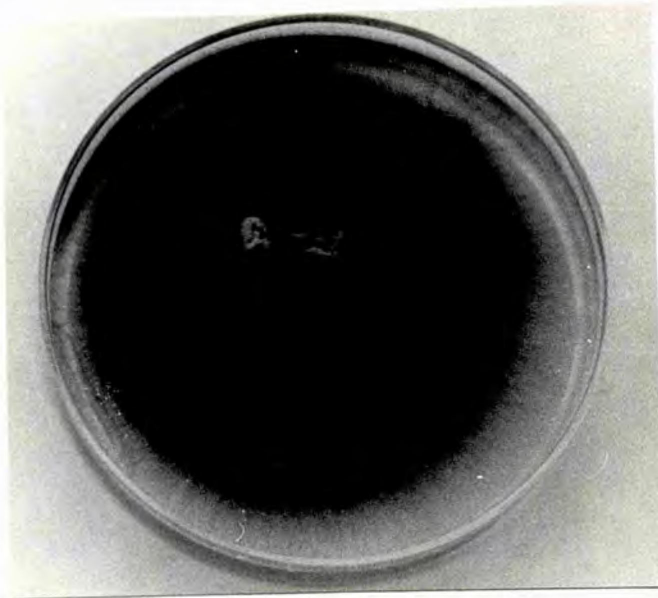
M8

Fig. 78. (cont.)

Continued .....



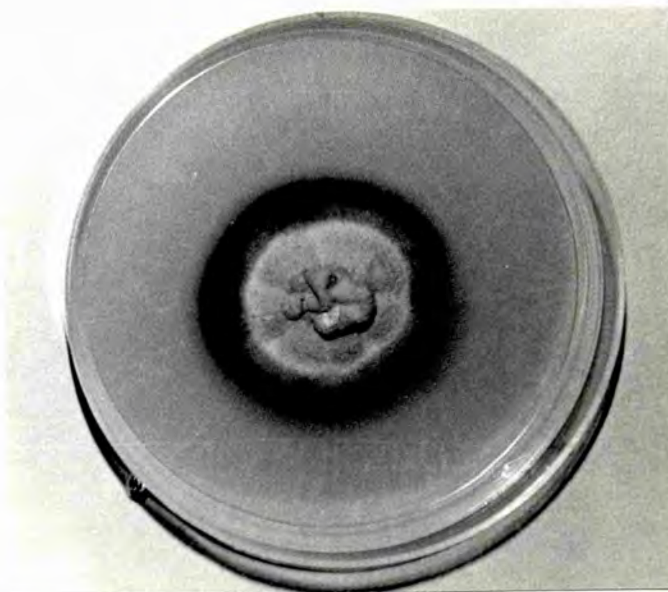
M13



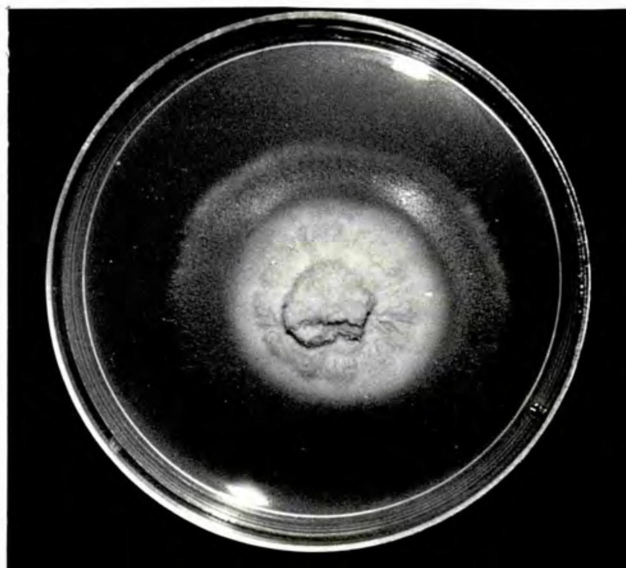
M23

Fig. 78. (cont.)

Continued .....



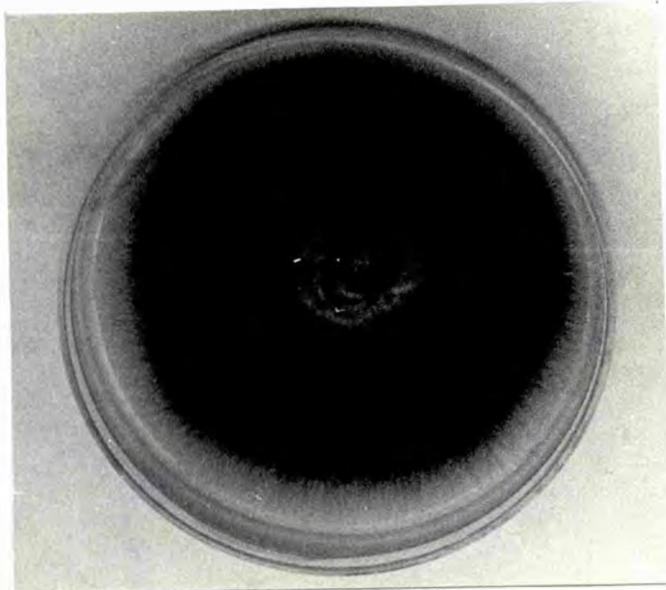
M37



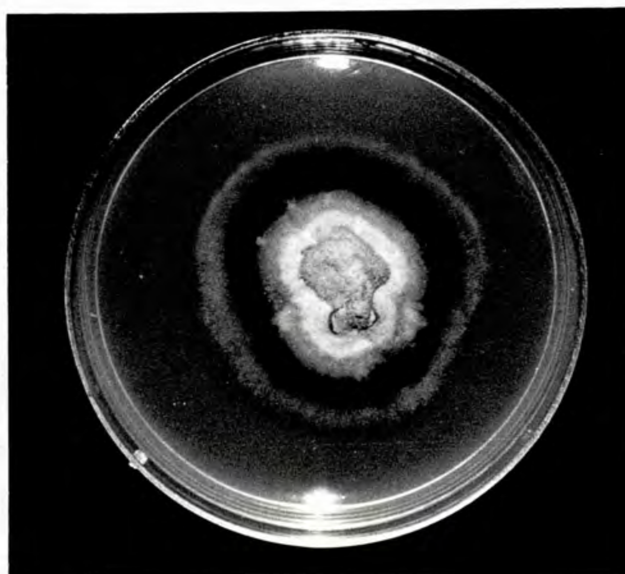
M44B

Fig. 78. (cont.)

Continued .....



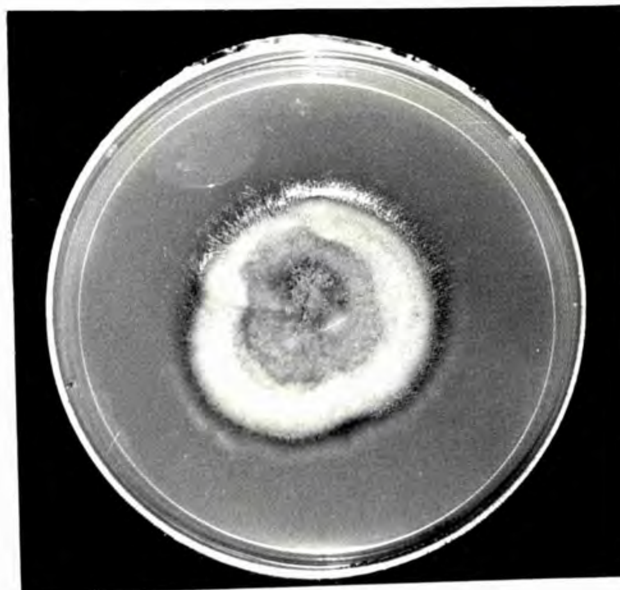
M48



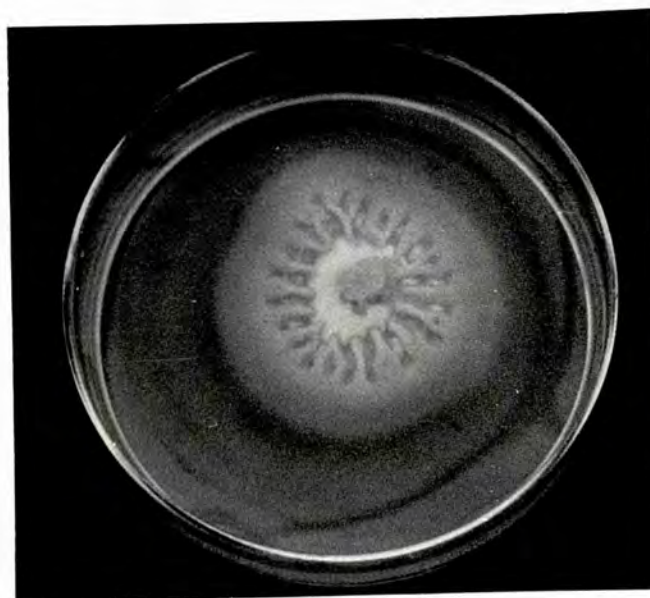
M65

Fig. 78. (cont.)

Continued .....



M70



M78
(*Verticillium psalliotae*)

Fig. 78. (cont.)

M3.

Pure white. Wide margin where the hyphae are adpressed to and penetrate the agar surface. Centre cream with patches of grey. Coarse aerial hyphae are also present.

Undersurface:- White margin to cream at the centre with patches of grey.

M7.

Pure white to cream at the centre with aerial hyphae. Hyphae are adpressed to and penetrate the agar surface at the margin.

Undersurface:- White margin to cream at the centre.

M8.

Pure white with patches of cream where the hyphae are adpressed to and penetrate the agar surface to form a dense mat. Centre cream with dark grey aerial hyphae (sparse).

Undersurface:- White margin with a cream centre.

M13.

Pure white. Hyphae adpressed to and penetrate agar surface at the margin. Aerial hyphae at the centre.

Undersurface:- White margin with a cream centre.

M23.

Jet black. Aerial hyphae grey and dense at the centre.

Undersurface:- Black at the margin, grey at the centre.

M37.

White margin, where dense hyphae are adpressed to and penetrate the agar surface. White mycelial mat folded at the centre, with a green margin. Extremely slow growing.

Undersurface:- White margin to greenish-cream at the centre.

M44B.

Pure white, hyphae adpressed to and penetrate agar surface. White fluffy aerial hyphae at the centre where the mycelial mat is wrinkled.

Undersurface:- White margin to green to cream at the centre.

M48.

Jet black. Hyphae penetrate agar surface at the margin. Grey hyphae adpressed to agar surface at the centre.

Undersurface:- Black margin to grey-black at the centre

M65.

Pure white to cream at the centre with patches of grey.

Undersurface:- White margin to greyish-cream at the centre.

M70.

Pure white with an irregular margin. Hyphae adpressed to and penetrate agar surface. Pinkish-brown centre with a yellowish-green margin.

Undersurface:- White margin which turns to yellow to dark green and finally to pink at the centre.

M78 (*Verticillum psalliotae* Treschow).

Pure white with a wide margin where hyphae are adpressed to and penetrate the agar surface. Dense hyphae on the surface of the agar at the centre.

Undersurface:- White margin to cream at the centre.

APPENDIX B

SYNTHESIS OF ECTOMYCORRHIZAE.

1. Fortin's Method

(Fortin, 1966)

Table 52.

The effect of substrate, fungus and medium on the lateral

Fungus	Substrate	Medium	Period of incubation (months)
No fungus (control)	Perlite	Mineral	3
No fungus (control)	Perlite	Mineral	8
No fungus (control)	Perlite	Dilute mineral	3
No fungus (control)	Perlite	Dilute mineral	8
M1	Perlite	Mineral	2
M2	Perlite	Mineral	2
M2	Perlite	Dilute mineral	3
M3	Perlite	Mineral	2
M3	Perlite	Mineral	2•5
M4	Perlite	Mineral	2
M4	Perlite	Mineral	3
M4	Perlite	Mineral	6
M4	Perlite	Dilute mineral	3
M4	Perlite	Dilute mineral	6
M4	Perlite	Dilute mineral	8

root growth of explants of Pinus sylvestris.

Mean number of laterals per root	Standard error	Mean length of laterals per root (mm)	Standard error	Number of replicates
1•4	(1•4)	2•7	(2•7)	5
1•0	(0•7)	2•2	(2•2)	5
1•0	(1•0)	1•2	(1•2)	5
1•6	(1•6)	1•9	(1•9)	5
1•4	(1•3)	5•8	(5•8)	10
0•0	(0•0)	0•0	(0•0)	9
0•6	(0•6)	0•7	(0•7)	10
0•8	(0•8)	3•2	(3•2)	5
0•4	(0•4)	0•4	(0•4)	5
0•0	(0•0)	0•0	(0•0)	9
0•8	(0•8)	1•4	(1•4)	5
0•5	(0•5)	0•8	(0•8)	4
1•7	(1•3)	3•3	(2•0)	10
3•5	(2•1)	1•5	(0•0)	2
1•8	(1•6)	3•8	(3•4)	5

Continued .....

Table 52. (cont.)

Fungus	Substrate	Medium	Period of incubation (months)
M7	Perlite	Mineral	3
M8	Perlite	Mineral	2
M9	Perlite	Mineral	3
M9	Perlite	Mineral	8
M9	Perlite	Dilute mineral	3
M9	Perlite	Dilute mineral	8
M10	Perlite	Mineral	2
M10	Perlite	Dilute mineral	3
M10	Perlite	Dilute mineral	7
M11	Perlite	Mineral	3
M11	Perlite	Mineral	8
M11	Perlite	Dilute mineral	4
M11	Perlite	Dilute mineral	8
M13	Perlite	Mineral	3
M13	Perlite	Mineral	7
M13	Perlite	Dilute mineral	3
M13	Perlite	Dilute mineral	9
M15	Perlite	Mineral	3
M15	Perlite	Mineral	7
M15	Perlite	Dilute mineral	3
M15	Perlite	Dilute mineral	9
M16	Perlite	Mineral	2

Mean number of laterals per root	Standard error	Mean length of laterals per root (mm)	Standard error	Number of replicates
1•3	(1•3)	2•4	(2•4)	7
0•3	(0•3)	0•6	(0•6)	10
1•6	(1•3)	3•8	(3•6)	5
1•0	(1•0)	0•2	(0•0)	5
0•0	(0•0)	0•0	(0•0)	5
2•0	(1•0)	5•1	(5•1)	5
0•3	(0•3)	0•6	(0•6)	10
0•8	(0•8)	0•6	(0•5)	5
0•5	(0•5)	1•0	(1•0)	2
1•0	(1•0)	0•8	(0•7)	5
1•2	(0•8)	3•7	(3•3)	5
2•2	(1•5)	4•1	(3•4)	5
1•4	(1•1)	3•6	(2•1)	5
0•5	(0•5)	1•0	(1•0)	5
1•4	(1•1)	1•0	(0•6)	5
0•8	(0•4)	0•2	(0•2)	5
2•0	(1•4)	2•7	(2•1)	5
1•0	(1•0)	0•5	(0•5)	5
2•0	(2•0)	0•8	(0•8)	5
1•4	(1•1)	2•4	(2•4)	5
1•8	(1•3)	4•3	(4•3)	5
0•0	(0•0)	0•0	(0•0)	10

Continued .....

Table 52. (cont.)

Fungus	Substrate	Medium	Period of incubation (months)
M21	Perlite	Mineral	3
M21	Perlite	Mineral	8
M21	Perlite	Dilute mineral	3
M21	Perlite	Dilute mineral	8
M23	Perlite	Mineral	3
M23	Perlite	Mineral	8
M23	Perlite	Dilute mineral	3
M23	Perlite	Dilute mineral	8
M28	Perlite	Mineral	5
M28	Perlite	Mineral	16
M28	Perlite	Dilute mineral	3
M28	Perlite	Dilute mineral	16
M32	Perlite	Mineral	15
M32	Perlite	Mineral	18
M32	Perlite	Dilute mineral	15
M32	Perlite	Dilute mineral	18
M8+M16	Perlite	Mineral	2
M1+M3	Perlite	Mineral	2
M37	Perlite	Mineral	12
M37	Perlite	Mineral	12•5
M37	Perlite	Dilute mineral	12
M37	Perlite	Dilute mineral	12

Mean number of laterals per root	Standard error	Mean length of laterals per root (mm)	Standard error	Number of replicates
0•4	(0•4)	0•6	(0•6)	5
0•4	(0•4)	2•6	(2•6)	5
0•0	(0•0)	0•0	(0•0)	5
0•8	(0•8)	0•7	(0•7)	5
0•0	(0•0)	0•0	(0•0)	5
1•0	(1•0)	0•3	(0•3)	5
0•2	(0•2)	0•1	(0•1)	5
1•0	(1•0)	0•4	(0•4)	5
0•6	(0•6)	1•1	(1•1)	5
1•8	(0•9)	2•5	(1•5)	5
0•4	(0•4)	0•2	(0•2)	5
0•0	(0•0)	0•0	(0•0)	5
2•0	(1•2)	2•0	(1•3)	5
1•0	(0•6)	1•5	(1•7)	5
1•2	(0•8)	0•7	(0•5)	5
2•2	(1•9)	1•4	(0•4)	4
0•2	(0•2)	0•3	(0•3)	10
0•1	(0•1)	0•1	(0•1)	8
0•0	(0•0)	0•0	(0•0)	5
0•2	(0•2)	0•2	(0•2)	5
0•2	(0•2)	0•2	(0•2)	5
0•0	(0•0)	0•0	(0•0)	4

Continued .....

Table 52. (cont.)

Fungus	Substrate	Medium	Period of incubation (months)
M44B	Perlite	Mineral	14
M44B	Perlite	Mineral	14
M44B	Perlite	Dilute mineral	13
M44B	Perlite	Dilute mineral	14
M48	Perlite	Mineral	14
M48	Perlite	Mineral	14
M48	Perlite	Dilute mineral	12
M48	Perlite	Dilute mineral	12
M65	Perlite	Mineral	14
M65	Perlite	Mineral	14
M65	Perlite	Dilute mineral	13
M65	Perlite	Dilute mineral	13
M70	Perlite	Mineral	14
M70	Perlite	Mineral	14
M70	Perlite	Dilute mineral	12
M70	Perlite	Dilute mineral	12
M78	Perlite	Mineral	12
M78	Perlite	Mineral	12
M78	Perlite	Dilute mineral	12
M78	Perlite	Dilute mineral	12
B.luteus	Perlite	Mineral	18
B.luteus	Perlite	Mineral	15
B.luteus	Perlite	Dilute mineral	4
B.luteus	Perlite	Dilute mineral	8

Mean number of laterals per root	Standard error	Mean length of laterals per root (mm)	Standard error	Number of replicates
0•6	(0•5)	1•4	(1•4)	5
0•8	(0•8)	3•2	(3•2)	5
0•2	(0•2)	0•2	(0•2)	5
0•0	(0•0)	0•0	(0•0)	5
0•2	(0•2)	0•6	(0•6)	5
0•0	(0•0)	0•0	(0•0)	5
0•6	(0•6)	0•2	(0•2)	5
0•0	(0•0)	0•0	(0•0)	4
0•4	(0•4)	0•8	(0•8)	5
0•0	(0•0)	0•0	(0•0)	5
0•2	(0•2)	0•2	(0•2)	5
0•4	(0•4)	0•3	(0•3)	5
0•2	(0•2)	0•2	(0•2)	5
0•0	(0•0)	0•0	(0•0)	5
0•0	(0•0)	0•0	(0•0)	5
0•0	(0•0)	0•0	(0•0)	5
0•6	(0•5)	0•7	(0•7)	5
0•2	(0•2)	1•0	(1•0)	5
1•8	(0•8)	1•7	(1•0)	5
1•6	(1•6)	0•8	(0•8)	5
2•5	(0•6)	6•4	(3•4)	4
1•8	(0•9)	2•6	(1•6)	4
2•5	(2•2)	2•1	(2•1)	6
2•5	(1•3)	3•9	(2•4)	4

Continued .....

Table 52. (cont.)

Fungus	Substrate	Medium	Period of incubation (months)
B.variegatus1	Perlite	Mineral	2
B.variegatus1	Perlite	Dilute mineral	3
B.variegatus1	Perlite	Dilute mineral	3
M1	Peat+perlite	Mineral	2
M3	Peat+perlite	Mineral	2
M7	Peat+perlite	Mineral	3
M16	Peat+perlite	Mineral	2
M1+M3	Peat+perlite	Mineral	2
M8+M16	Peat+perlite	Mineral	2
M1	Peat+humus	Mineral	2
M3	Peat+humus	Mineral	2
M3	Peat+humus	Mineral	2
M7	Peat+humus	Mineral	1•5
M7	Peat+humus	Mineral	2
M8	Peat+humus	Mineral	2
M16	Peat+humus	Mineral	2

Mean number of laterals per root	Standard error	Mean length of laterals per root (mm)	Standard error	Number of replicates
0•7	(0•7)	2•0	(2•0)	10
1•4	(1•3)	2•4	(2•4)	5
2•6	(1•1)	4•7	(4•5)	5
0•0	(0•0)	0•0	(0•0)	6
0•1	(0•1)	0•1	(0•1)	10
0•0	(0•0)	0•0	(0•0)	5
0•3	(0•3)	0•6	(0•6)	10
1•0	(1•0)	0•5	(0•5)	4
0•3	(0•3)	0•4	(0•4)	10
0•0	(0•0)	0•0	(0•0)	9
0•2	(0•2)	0•2	(0•2)	5
0•4	(0•4)	0•6	(0•6)	5
0•3	(0•3)	1•0	(1•0)	4
0•0	(0•0)	0•0	(0•0)	5
0•5	(0•5)	0•3	(0•3)	9
0•1	(0•1)	0•2	(0•2)	10

Continued .....

Table 52. (cont.)

Fungus	Substrate	Medium	Period of incubation (months)
M1	Sand+peat	Mineral	2
M3	Sand+peat	Mineral	1·5
M7	Sand+peat	Mineral	2
M8	Sand+peat	Mineral	2
M16	Sand+peat	Mineral	2
M1	Peat	Mineral	2
M3	Peat	Mineral	2
M3	Peat	Mineral	2
M7	Peat	Mineral	2
M8	Peat	Mineral	2
M16	Peat	Mineral	2
M1	Humus	Mineral	2
M3	Humus	Mineral	2
M3	Humus	Mineral	2
M3	Humus	Mineral	2
M7	Humus	Mineral	2
M16	Humus	Mineral	2

Mean number of laterals per root	Standard error	Mean length of laterals per root (mm)	Standard error	Number of replicates
0•4	(0•4)	0•4	(0•4)	9
0•0	(0•0)	0•0	(0•0)	9
0•2	(0•2)	0•4	(0•4)	10
0•0	(0•0)	0•0	(0•0)	10
0•0	(0•0)	0•0	(0•0)	7
0•3	(0•3)	0•9	(0•9)	9
0•0	(0•0)	0•0	(0•0)	6
1•0	(1•0)	0•5	(0•5)	5
0•0	(0•0)	0•0	(0•0)	7
0•2	(0•2)	0•4	(0•4)	5
0•0	(0•0)	0•0	(0•0)	10
0•0	(0•0)	0•0	(0•0)	4
0•0	(0•0)	0•0	(0•0)	3
0•4	(0•4)	0•3	(0•3)	4
0•0	(0•0)	0•0	(0•0)	2
0•2	(0•2)	0•6	(0•5)	5
0•1	(0•1)	0•1	(0•1)	8

Continued .....

Table 52. (cont.)

Fungus	Substrate	Medium	Period of incubation (months)
M1	Humus	Distilled water	2
M3	Humus	Distilled water	2
M3	Humus	Distilled water	2
M7	Humus	Distilled water	2
M8	Humus	Distilled water	2
M3	Non-sterile humus	Mineral	2
M3	Non-sterile humus	Mineral	2
M23	Non-sterile humus	Mineral	2
M23	non-sterile humus	Mineral+benomyl	2

Mean number of laterals per root	Standard error	Mean length of laterals per root (mm)	Standard error	Number of replicates
0•0	(0•0)	0•0	(0•0)	5
0•0	(0•0)	0•0	(0•0)	2
0•0	(0•0)	0•0	(0•0)	3
0•0	(0•0)	0•0	(0•0)	3
0•0	(0•0)	0•0	(0•0)	4
0•0	(0•0)	0•0	(0•0)	3
0•0	(0•0)	0•0	(0•0)	4
0•2	(0•2)	0•2	(0•2)	10
0•0	(0•0)	0•0	(0•0)	9

APPENDIX C

EXPERIMENTS WITH EXCISED TOMATO ROOTS.

1. Experiments with live and killed excised tomato roots.

a) Growth of various isolates in the presence of excised tomato roots in MLM.

Table 53.

Growth of root isolates and Boletus variegatus1 with live and killed excised tomato roots in MLM.

Fungus	Treatment	Period of incubation	Mean dry weight (mg)	Standard error	P
M1	No root	7 days	19•8	(2•3)	-
M1	+Live root	7 days	23•4	(0•9)	0•02
M1	+Killed root	7 days	20•0	(4•4)	n.s.
M2	No root	7 days	16•6	(5•4)	-
M2	+Live root	7 days	18•3	(4•2)	n.s.
M2	+Killed root	7 days	18•3	(4•2)	n.s.
M3	No root	7 days	18•1	(5•2)	-
M3	+Live root	7 days	26•5	(3•6)	0•05
M3	+Killed root	7 days	26•8	(3•7)	0•05
M4	No root	7 days	22•6	(1•9)	-
M4	+Live root	7 days	31•6	(5•6)	0•02
M4	+Killed root	7 days	27•7	(3•5)	0•05
M5	No root	7 days	11•5	(4•1)	-
M5	+Live root	7 days	12•6	(0•4)	n.s.
M5	+Killed root	7 days	15•2	(1•1)	0•02
M6	No root	7 days	7•5	(2•0)	-
M6	+Live root	7 days	15•0	(0•9)	0•001
M6	+Killed root	7 days	6•1	(1•6)	n.s.

Continued .....

Table 53. (cont.)

Fungus	Treatment	Period of incubation	Mean dry weight (mg)	Standard error	P
M7	No root	7 days	10•6	(3•2)	–
M7	+Live root	7 days	19•0	(0•6)	0•002
M7	+Killed root	7 days	12•5	(2•0)	n.s.
M8	No root	7 days	13•1	(3•3)	–
M8	+Live root	7 days	19•2	(5•2)	n.s.
M8	+Killed root	7 days	15•2	(3•7)	n.s.
M9	No root	7 days	19•5	(0•7)	–
M9	+Live root	7 days	29•6	(6•0)	0•02
M9	+Killed root	7 days	18•2	(1•5)	n.s.
M10	No root	7 days	17•6	(1•9)	–
M10	+Live root	7 days	15•1	(2•0)	n.s.
M10	+Killed root	7 days	17•3	(2•4)	n.s.
M11	No root	7 days	15•8	(1•9)	–
M11	+Live root	7 days	24•4	(3•2)	0•01
M11	+Killed root	7 days	13•3	(2•1)	n.s.
M13	No root	7 days	31•4	(5•9)	–
M13	+Live root	7 days	83•3	(11•0)	0•001
M13	+Killed root	7 days	56•7	(16•6)	0•02
M13	No root	14 days	51•3	(7•0)	–
M13	+Live root	14 days	221•8	(55•5)	0•002
M13	+Killed root	14 days	109•5	(13•1)	0•001
B.variegatus1	No root	7 days	0•0	(0•0)	–
B.variegatus1	+Live root	7 days	3•7	(1•9)	0•01
B.variegatus1	+Killed root	7 days	0•0	(0•0)	n.s.

Continued .....

Table 53. (cont.)

Fungus	Treatment	Period of incubation	Mean dry weight (mg)	Standard error	P
B.variegatus1	No root	14 days	11•7	(3•1)	–
B.variegatus1	+Live root	14 days	31•4	(15•2)	0•05
B.variegatus1	No root	14 days	14•3	(8•3)	–
B.variegatus1	+Killed root	14 days	7•6	(3•4)	n.s.
M15	No root	7 days	13•4	(2•1)	–
M15	+Live root	7 days	18•5	(4•1)	0•05
M15	+Killed root	7 days	13•8	(0•3)	n.s.
M16	No root	7 days	13•7	(2•9)	–
M16	+Live root	7 days	18•6	(0•7)	0•02
M16	+Killed root	7 days	13•2	(1•2)	n.s.
M16	No root	7 days	12•1	(2•3)	–
M16	+Live root*	7 days	29•4	(3•5)	0•001
M16	+Killed root*	7 days	15•8	(2•0)	0•02
M21	No root	7 days	24•6	(1•3)	–
M21	+Live root	7 days	36•0	(5•7)	0•01
M21	+Killed root	7 days	24•1	(0•8)	n.s.
M22	No root	7 days	32•3	(5•9)	–
M22	+Live root	7 days	33•7	(10•4)	n.s.
M22	+Killed root	7 days	39•4	(2•6)	n.s.
M23	No root	7 days	38•3	(2•7)	–
M23	+Live root	7 days	82•2	(16•4)	0•001
M23	+Killed root	7 days	83•1	(14•3)	0•002
M37	No root	7 days	31•5	(3•6)	–
M37	+Live root	7 days	39•1	(2•8)	0•02
M37	+Killed root	7 days	29•1	(8•3)	n.s.

Continued .....

Table 53. (cont.)

Fungus	Treatment	Period of incubation	Mean dry weight (mg)	Standard error	P
M37	No root	14 days	58•2	(7•1)	-
M37	+Live root	14 days	188•2	(17•0)	0•001
M37	+Killed root	14 days	155•9	(15•1)	0•001
M44B	No root	7 days	20•3	(0•7)	-
M44B	+Live root	7 days	28•4	(0•7)	0•001
M44B	+Killed root	7 days	18•6	(2•7)	n.s.
M44B	No root	14 days	54•4	(6•6)	-
M44B	+Live root	14 days	154•0	(23•9)	0•001
M44B	+Killed root	14 days	105•8	(8•4)	0•001
M48	No root	7 days	43•4	(4•3)	-
M48	+Live root	7 days	92•1	(19•6)	0•01
M48	+Killed root	7 days	65•2	(8•6)	0•01
M48	No root	14 days	258•6	(36•4)	-
M48	+Live root	14 days	344•4	(5•1)	0•01
M48	+Killed root	14 days	320•6	(15•2)	0•02
M65	No root	7 days	39•0	(6•0)	-
M65	+Live root	7 days	42•0	(5•0)	n.s.
M65	+Killed root	7 days	39•0	(2•5)	n.s.
M65	No root	14 days	74•3	(10•3)	-
M65	+Live root	14 days	234•7	(33•3)	0•001
M65	+Killed root	14 days	142•8	(23•3)	0•01
M70	No root	7 days	32•2	(3•1)	-
M70	+Live root	7 days	28•9	(5•1)	n.s.
M70	+Killed root	7 days	27•6	(2•3)	n.s.

Continued .....

Table 53. (cont.)

Fungus	Treatment	Period of incubation	Mean dry weight (mg)	Standard error	P
M70	No root	14 days	86•5	(35•4)	–
M70	+Live root	14 days	131•9	(4•3)	0•05
M70	+Killed root	14 days	92•6	(10•1)	n.s.
M78	No root	7 days	119•2	(32•6)	–
M78	+Live root	7 days	183•3	(27•4	0•02
M78	+Killed root	7 days	100•0	(8•7)	0•01
M78	No root	14 days	202•4	(5•9)	–
M78	+Live root	14 days	222•9	(18•5)	0•05
M78	+Killed root	14 days	218•3	(20•5)	n.s.

* 14 day old excised tomato roots (Clone A) were used.

APPENDIX D

Chromatographic bioassay of excised tomato root preparations and pine seedling root exudates.

Table 54.

Growth of M13 in MLM with different Rf fractions after the fractionation of live excised tomato root preparations, by paper chromatography.

Solvent	Root preparation	Method of sterilization of the fractions	Rf fraction	Mean dry weight (mg)	Standard error	P
BuA	Exudate	UV	No exudate (control)	104•1	(11•6)	–
BuA	Exudate	UV	1	102•7	(11•0)	n.s.
BuA	Exudate	UV	2	122•3	(14•2)	n.s.
BuA	Exudate	UV	3	131•3	(18•5)	0•05
BuA	Exudate	UV	4	140•6	(9•0)	0•01
BuA	Exudate	UV	5	109•1	(6•8)	n.s.
BuA	Exudate	UV	6	120•2	(1•6)	0•05
BuA	Exudate	UV	7	105•0	(5•6)	n.s.
BuA	Exudate	UV	8	139•7	(45•0)	n.s.
BuA	Exudate	UV	9	98•5	(4•5)	n.s.
BuA	Exudate	UV	10	116•2	(12•2)	n.s.
BuA	Exudate	Autoclaving	No exudate (control)	74•9	(4•7)	–
BuA	Exudate	Autoclaving	1	86•1	(6•0)	0•02
BuA	Exudate	Autoclaving	2	94•7	(2•6)	0•001
BuA	Exudate	Autoclaving	3	100•3	(6•5)	0•002
BuA	Exudate	Autoclaving	4	108•9	(6•9)	0•001
BuA	Exudate	Autoclaving	5	93•1	(8•2)	0•01
BuA	Exudate	Autoclaving	6	87•4	(1•6)	0•01
BuA	Exudate	Autoclaving	7	86•1	(2•4)	0•01
BuA	Exudate	Autoclaving	8	90•3	(5•1)	0•01
BuA	Exudate	Autoclaving	9	86•4	(11•9)	n.s.
BuA	Exudate	Autoclaving	10	83•8	(11•8)	n.s.

Continued .....

Table 54. (cont.)

Solvent	Root preparation	Method of sterilization of the fractions	Rf fraction	Mean dry weight (mg)	Standard error	P
BuP	Exudate	UV	No exudate (control)	126•6	(5•5)	–
BuP	Exudate	UV	1	147•0	(2•8)	0•01
BuP	Exudate	UV	2	130•1	(4•3)	n.s.
BuP	Exudate	UV	3	144•2	(3•3)	0•002
BuP	Exudate	UV	4	132•5	(5•1)	n.s.
BuP	Exudate	UV	5	120•8	(19•8)	n.s.
BuP	Exudate	UV	6	120•7	(23•2)	n.s.
BuP	Exudate	UV	7	126•1	(17•5)	n.s.
BuP	Exudate	UV	8	136•5	(9•5)	n.s.
BuP	Exudate	UV	9	148•5	(10•7)	0•01
BuP	Exudate	UV	10	131•7	(1•3)	n.s.
BuP	Exudate	Autoclaving	No exudate (control)	73•8	(11•9)	–
BuP	Exudate	Autoclaving	1	83•6	(6•3)	n.s.
BuP	Exudate	Autoclaving	2	76•6	(8•4)	n.s.
BuP	Exudate	Autoclaving	3	74•2	(6•8)	n.s.
BuP	Exudate	Autoclaving	4	75•3	(8•4)	n.s.
BuP	Exudate	Autoclaving	5	77•1	(5•1)	n.s.
BuP	Exudate	Autoclaving	6	80•4	(6•4)	n.s.
BuP	Exudate	Autoclaving	7	76•8	(8•1)	n.s.
BuP	Exudate	Autoclaving	8	80•8	(9•4)	n.s.
BuP	Exudate	Autoclaving	9	79•3	(5•2)	n.s.
BuP	Exudate	Autoclaving	10	73•6	(10•1)	n.s.

Continued .....

Table 54. (cont.)

Solvent	Root preparation	Method of sterilization of the fractions	Rf fraction	Mean dry weight (mg)	Standard error	P
BuA	†Dialysate	UV	No dialysate (control)	74•9	(4•7)	-
BuA	Dialysate	UV	1	86•1	(6•0)	0•02
BuA	Dialysate	UV	2	95•0	(2•8)	0•002
BuA	Dialysate	UV	3	100•3	(6•5)	0•002
BuA	Dialysate	UV	4	107•2	(4•1)	0•001
BuA	Dialysate	UV	5	93•1	(8•2)	0•01
BuA	Dialysate	UV	6	87•4	(1•6)	0•01
BuA	Dialysate	UV	7	86•1	(2•4)	0•01
BuA	Dialysate	UV	8	90•4	(5•1)	0•01
BuA	Dialysate	UV	9	86•5	(12•1)	n.s.
BuA	Dialysate	UV	10	83•8	(11•8)	n.s.
BuA	Dialysate	Autoclaving	No dialysate (control)	87•1	(7•4)	-
BuA	Dialysate	Autoclaving	1	80•2	(2•8)	n.s.
BuA	Dialysate	Autoclaving	2	74•5	(9•1)	0•05*
BuA	Dialysate	Autoclaving	3	95•0	(16•3)	n.s.
BuA	Dialysate	Autoclaving	4	84•3	(16•8)	n.s.
BuA	Dialysate	Autoclaving	5	87•2	(19•6)	n.s.
BuA	Dialysate	Autoclaving	6	85•6	(7•2)	n.s.
BuA	Dialysate	Autoclaving	7	81•5	(4•7)	n.s.
BuA	Dialysate	Autoclaving	8	83•3	(11•3)	n.s.
BuA	Dialysate	Autoclaving	9	78•1	(12•4)	n.s.
BuA	Dialysate	Autoclaving	10	84•7	(13•9)	n.s.

Continued .....

Table 54. (cont.)

Solvent	Root preparation	Method of sterilization of the fractions	Rf fraction	Mean dry weight (mg)	Standard error	P
BuA	Extract	UV	No extract (control)	86•2	(6•0)	–
BuA	Extract	UV	1	121•7	(21•1)	0•02
BuA	Extract	UV	2	130•3	(4•7)	0•001
BuA	Extract	UV	3	133•2	(9•2)	0•001
BuA	Extract	UV	4	134•5	(14•9)	0•002
BuA	Extract	UV	5	89•9	(7•7)	n.s.
BuA	Extract	UV	6	94•7	(11•0)	n.s.
BuA	Extract	UV	7	89•0	(8•9)	n.s.
BuA	Extract	UV	8	115•7	(23•5)	0•05
BuA	Extract	UV	9	98•7	(24•4)	n.s.
BuA	Extract	UV	10	111•8	(16•4)	0•02
BuA	Extract	Autoclaving	No extract (control)	123•7	(29•0)	–
BuA	Extract	Autoclaving	1	154•4	(18•9)	n.s.
BuA	Extract	Autoclaving	No extract (control)	91•1	(10•8)	–
BuA	Extract	Autoclaving	2	205•9	(65•9)	0•01
BuA	Extract	Autoclaving	3	202•7	(55•8)	0•01
BuA	Extract	Autoclaving	4	127•9	(11•1)	0•01
BuA	Extract	Autoclaving	5	111•5	(7•3)	0•02
BuA	Extract	Autoclaving	6	92•7	(10•1)	n.s.
BuA	Extract	Autoclaving	7	109•2	(47•5)	n.s.
BuA	Extract	Autoclaving	8	117•7	(32•8)	n.s.
BuA	Extract	Autoclaving	9	83•0	(6•3)	n.s.
BuA	Extract	Autoclaving	10	86•8	(2•2)	n.s.

Continued .....

Table 54. (cont.)

Solvent	Root preparation	Method of sterilization of the fractions	Rf fraction	Mean dry weight (mg)	Standard error	P
BuP	Extract	UV	No extract (control)	85•7	(22•9)	–
BuP	Extract	UV	1	132•2	(2•6)	0•01
BuP	Extract	UV	2	100•0	(4•2)	n.s.
BuP	Extract	UV	3	106•3	(13•8)	n.s.
BuP	Extract	UV	4	104•5	(15•6)	n.s.
BuP	Extract	UV	5	81•9	(6•9)	n.s.
BuP	Extract	UV	6	84•9	(8•7)	n.s.
BuP	Extract	UV	7	76•0	(12•6)	n.s.
BuP	Extract	UV	8	84•8	(5•7)	n.s.
BuP	Extract	UV	9	54•1	(11•1)	0•05*
BuP	Extract	UV	10	81•3	(4•5)	n.s.
BuP	Extract	Autoclaving	No extract (control)	71•7	(12•0)	–
BuP	Extract	Autoclaving	1	121•7	(3•7)	0•001
BuP	Extract	Autoclaving	2	64•0	(10•9)	n.s.
BuP	Extract	Autoclaving	3	67•5	(7•3)	n.s.
BuP	Extract	Autoclaving	4	78•9	(8•3)	n.s.
BuP	Extract	Autoclaving	5	65•5	(18•8)	n.s.
BuP	Extract	Autoclaving	6	65•8	(14•8)	n.s.
BuP	Extract	Autoclaving	7	84•0	(6•4)	n.s.
BuP	Extract	Autoclaving	8	87•7	(17•3)	n.s.
BuP	Extract	Autoclaving	9	62•2	(3•8)	n.s.
BuP	Extract	Autoclaving	10	59•6	(5•2)	n.s.

Continued .....

Table 54. (cont.)

Solvent	Root preparation	Method of sterilization of the fractions	Rf fraction	Mean dry weight (mg)	Standard error	P
BuA	Homogenate	UV	No homogenate (control)	119•5	(16•3)	–
BuA	Homogenate	UV	1	110•3	(31•2)	n.s.
BuA	Homogenate	UV	2	125•1	(20•0)	n.s.
BuA	Homogenate	UV	3	158•2	(9•0)	0•01
BuA	Homogenate	UV	4	141•2	(25•4)	n.s.
BuA	Homogenate	UV	5	111•7	(6•6)	n.s.
BuA	Homogenate	UV	6	115•8	(6•2)	n.s.
BuA	Homogenate	UV	7	122•2	(14•6)	n.s.
BuA	Homogenate	UV	8	108•2	(10•6)	n.s.
BuA	Homogenate	UV	9	125•5	(16•2)	n.s.
BuA	Homogenate	UV	10	142•6	(7•4)	0•05
BuA	Homogenate	Autoclaving	No homogenate (control)	70•7	(8•3)	–
BuA	Homogenate	Autoclaving	1	93•9	(12•8)	0•02
BuA	Homogenate	Autoclaving	2	100•3	(13•7)	0•01
BuA	Homogenate	Autoclaving	3	112•8	(6•6)	0•001
BuA	Homogenate	Autoclaving	4	105•9	(8•7)	0•002
BuA	Homogenate	Autoclaving	5	87•9	(14•7)	n.s.
BuA	Homogenate	Autoclaving	6	81•1	(18•1)	n.s.
BuA	Homogenate	Autoclaving	7	72•9	(26•4)	n.s.
BuA	Homogenate	Autoclaving	8	66•6	(8•0)	n.s.
BuA	Homogenate	Autoclaving	9	75•8	(12•6)	n.s.
BuA	Homogenate	Autoclaving	10	69•6	(10•5)	n.s.

Continued .....

Table 54. (cont.)

Solvent	Root preparation	Method of sterilization of the fractions	Rf fraction	Mean dry weight (mg)	Standard error	P
BuP	Homogenate	UV	No homogenate (control)	114•5	(33•5)	-
BuP	Homogenate	UV	1	192•6	(64•9)	n.s.
BuP	Homogenate	UV	2	121•8	(31•5)	n.s.
BuP	Homogenate	UV	3	128•0	(10•1)	n.s.
BuP	Homogenate	UV	4	126•8	(23•8)	n.s.
BuP	Homogenate	UV	5	104•6	(30•5)	n.s.
BuP	Homogenate	UV	6	99•0	(17•1)	n.s.
BuP	Homogenate	UV	7	116•9	(37•6)	n.s.
BuP	Homogenate	UV	8	84•7	(9•8)	n.s.
BuP	Homogenate	UV	9	74•4	(9•2)	0•05*
BuP	Homogenate	UV	10	93•0	(22•8)	n.s.
BuP	Homogenate	Autoclaving	No homogenate (control)	72•5	(1•6)	-
BuP	Homogenate	Autoclaving	1	129•1	(8•2)	0•001
BuP	Homogenate	Autoclaving	2	75•9	(2•2)	0•05
BuP	Homogenate	Autoclaving	3	78•1	(5•5)	n.s.
BuP	Homogenate	Autoclaving	4	71•9	(7•0)	n.s.
BuP	Homogenate	Autoclaving	5	85•2	(4•5)	0•01
BuP	Homogenate	Autoclaving	6	70•1	(5•2)	n.s.
BuP	Homogenate	Autoclaving	7	102•2	(15•5)	0•001
BuP	Homogenate	Autoclaving	8	76•1	(5•9)	n.s.
BuP	Homogenate	Autoclaving	9	71•8	(5•0)	n.s.
BuP	Homogenate	Autoclaving	10	71•7	(5•7)	n.s.

* Inhibition

† Dialysate collected in distilled water from live exuding roots enclosed within dialysis membrane bags.

Table 55.

Growth of Boletus variegatus1 in MLM with different Rf fractions after the fractionation of live excised tomato root preparations, by paper chromatography.

Solvent	Root preparation	Method of sterilization of the fractions	Rf fraction	Mean dry weight (mg)	Standard error	P
BuA	Exudate	UV	No exudate (control)	9·5	(3·3)	–
BuA	Exudate	UV	1	2·2	(1·5)	0·01*
BuA	Exudate	UV	2	6·9	(3·7)	n.s.
BuA	Exudate	UV	3	3·3	(2·0)	0·02*
BuA	Exudate	UV	4	4·8	(0·6)	0·05*
BuA	Exudate	UV	5	2·4	(1·6)	0·01*
BuA	Exudate	UV	6	4·7	(1·0)	0·02*
BuA	Exudate	UV	7	6·7	(0·3)	n.s.
BuA	Exudate	UV	8	5·7	(0·5)	0·05*
BuA	Exudate	UV	9	7·8	(2·1)	n.s.
BuA	Exudate	UV	10	8·0	(1·3)	n.s.
BuA	Exudate	Autoclaving	No exudate (control)	10·3	(3·0)	–
BuA	Exudate	Autoclaving	1	6·1	(2·4)	0·05*
BuA	Exudate	Autoclaving	2	7·3	(1·7)	n.s.
BuA	Exudate	Autoclaving	3	7·2	(1·7)	n.s.
BuA	Exudate	Autoclaving	4	5·8	(1·9)	0·05*
BuA	Exudate	Autoclaving	5	6·7	(1·7)	0·05*
BuA	Exudate	Autoclaving	6	8·7	(0·8)	n.s.
BuA	Exudate	Autoclaving	7	6·9	(1·6)	n.s.
BuA	Exudate	Autoclaving	8	9·8	(3·5)	n.s.
BuA	Exudate	Autoclaving	9	12·7	(5·6)	n.s.
BuA	Exudate	Autoclaving	10	6·2	(1·5)	0·05*

Continued .....

Table 55. (cont.)

Solvent	Root preparation	Method of sterilization of the fractions	Rf fraction	Mean dry weight (mg)	Standard error	P
BuP	Exudate	UV	No exudate (control)	19•7	(7•3)	-
BuP	Exudate	UV	1	4•1	(1•7)	0•01[*]
BuP	Exudate	UV	2	12•9	(1•1)	n.s.
BuP	Exudate	UV	3	12•2	(1•6)	n.s.
BuP	Exudate	UV	4	11•6	(5•4)	n.s.
BuP	Exudate	UV	5	11•1	(1•0)	0•05[*]
BuP	Exudate	UV	6	13•6	(7•8)	n.s.
BuP	Exudate	UV	7	7•4	(2•6)	0•02[*]
BuP	Exudate	UV	8	6•7	(1•2)	0•01[*]
BuP	Exudate	UV	9	7•8	(5•0)	0•02[*]
BuP	Exudate	UV	10	14•8	(9•5)	n.s.
BuP	Exudate	Autoclaving	No exudate (control)	8•3	(1•2)	-
BuP	Exudate	Autoclaving	1	4•6	(3•2)	n.s.
BuP	Exudate	Autoclaving	2	7•0	(0•2)	n.s.
BuP	Exudate	Autoclaving	3	3•7	(2•3)	0•02[*]
BuP	Exudate	Autoclaving	4	6•6	(3•6)	n.s.
BuP	Exudate	Autoclaving	5	5•1	(1•3)	0•01[*]
BuP	Exudate	Autoclaving	6	6•0	(0•5)	0•05[*]
BuP	Exudate	Autoclaving	7	10•8	(4•4)	n.s.
BuP	Exudate	Autoclaving	8	6•3	(2•5)	n.s.
BuP	Exudate	Autoclaving	9	6•1	(0•9)	n.s.
BuP	Exudate	Autoclaving	10	7•4	(2•8)	n.s.

Continued .....

Table 55. (cont.)

Solvent	Root preparation	Method of sterilization of the fractions	Rf fraction	Mean dry weight (mg)	Standard error	P
BuA	Extract	UV	No extract (control)	28•2	(5•7)	-
BuA	Extract	UV	1	9•8	(3•7)	0•002*
BuA	Extract	UV	2	21•2	(13•1)	n.s.
BuA	Extract	UV	3	9•2	(0•9)	0•001*
BuA	Extract	UV	4	13•0	(2•7)	0•01
BuA	Extract	UV	5	17•5	(10•7)	n.s.
BuA	Extract	UV	6	27•2	(13•2)	n.s.
BuA	Extract	UV	7	18•5	(3•4)	0•02*
BuA	Extract	UV	8	12•4	(6•4)	0•01*
BuA	Extract	UV	9	17•1	(8•9)	0•05*
BuA	Extract	UV	10	27•1	(5•5)	n.s.
BuA	Extract	Autoclaving	No extract (control)	7•4	(2•0)	-
BuA	Extract	Autoclaving	1	7•4	(1•4)	n.s.
BuA	Extract	Autoclaving	2	6•7	(2•0)	n.s.
BuA	Extract	Autoclaving	3	8•3	(4•7)	n.s.
BuA	Extract	Autoclaving	4	7•0	(0•2)	n.s.
BuA	Extract	Autoclaving	5	5•3	(1•9)	n.s.
BuA	Extract	Autoclaving	6	7•1	(2•3)	n.s.
BuA	Extract	Autoclaving	7	7•3	(1•8)	n.s.
BuA	Extract	Autoclaving	8	6•8	(1•2)	n.s.
BuA	Extract	Autoclaving	9	5•2	(1•0)	0•05*
BuA	Extract	Autoclaving	10	8•9	(0•9)	n.s.

Continued .....

Table 55. (cont.)

Solvent	Root preparation	Method of sterilization of the fractions	Rf fraction	Mean dry weight (mg)	Standard error	P
BuP	Extract	UV	No extract (control)	16•5	(0•4)	–
BuP	Extract	UV	1	7•3	(2•7)	0•002*
BuP	Extract	UV	2	10•0	(3•8)	0•02*
BuP	Extract	UV	3	8•8	(1•2)	0•001*
BuP	Extract	UV	4	7•4	(1•5)	0•001*
BuP	Extract	UV	5	10•6	(1•4)	0•001*
BuP	Extract	UV	6	14•6	(4•4)	n.s.
BuP	Extract	UV	7	13•5	(2•9)	0•05*
BuP	Extract	UV	8	9•8	(1•3)	0•001*
BuP	Extract	UV	9	12•0	(2•8)	0•02*
BuP	Extract	UV	10	10•5	(0•2)	0•001*
BuP	Extract	Autoclaving	No extract (control)	14•3	(6•3)	–
BuP	Extract	Autoclaving	1	8•3	(0•4)	n.s.
BuP	Extract	Autoclaving	2	9•6	(4•5)	n.s.
BuP	Extract	Autoclaving	3	7•0	(1•5)	0•05*
BuP	Extract	Autoclaving	4	8•1	(4•0)	n.s.
BuP	Extract	Autoclaving	5	9•5	(6•5)	n.s.
BuP	Extract	Autoclaving	6	10•6	(0•7)	n.s.
BuP	Extract	Autoclaving	7	9•4	(5•1)	n.s.
BuP	Extract	Autoclaving	8	10•7	(4•0)	n.s.
BuP	Extract	Autoclaving	9	8•9	(1•9)	n.s.
BuP	Extract	Autoclaving	10	10•3	(1•8)	n.s.

Continued .....

Table 55. (cont.)

Solvent	Root preparation	Method of sterilization of the fractions	Rf fraction	Mean dry weight (mg)	Standard error	P
BuA	Homogenate	UV	No homogenate (control)	10•1	(1•5)	–
BuA	Homogenate	UV	1	5•1	(2•4)	0•01*
BuA	Homogenate	UV	2	7•5	(1•3)	0•01*
BuA	Homogenate	UV	3	9•9	(0•9)	n.s.
BuA	Homogenate	UV	4	6•8	(1•0)	0•02*
BuA	Homogenate	UV	5	6•6	(2•7)	0•05*
BuA	Homogenate	UV	6	10•2	(0•3)	n.s.
BuA	Homogenate	UV	7	6•4	(0•5)	0•01*
BuA	Homogenate	UV	8	7•7	(2•5)	n.s.
BuA	Homogenate	UV	9	10•7	(0•7)	n.s.
BuA	Homogenate	UV	10	9•3	(2•9)	n.s.
BuA	Homogenate	Autoclaving	No homogenate (control)	8•9	(5•1)	–
BuA	Homogenate	Autoclaving	1	6•3	(4•0)	n.s.
BuA	Homogenate	Autoclaving	2	5•2	(4•5)	n.s.
BuA	Homogenate	Autoclaving	3	4•0	(3•7)	n.s.
BuA	Homogenate	Autoclaving	4	3•0	(1•1)	0•05*
BuA	Homogenate	Autoclaving	5	5•3	(0•9)	n.s.
BuA	Homogenate	Autoclaving	6	8•3	(2•9)	n.s.
BuA	Homogenate	Autoclaving	7	6•2	(0•3)	n.s.
BuA	Homogenate	Autoclaving	8	7•8	(2•3)	n.s.
BuA	Homogenate	Autoclaving	9	3•1	(2•5)	0•05*
BuA	Homogenate	Autoclaving	10	4•4	(1•9)	n.s.

Continued .....

Table 55. (cont.)

Solvent	Root preparation	Method of sterilization of the fractions	Rf fraction	Mean dry weight (mg)	Standard error	P
BuP	Homogenate	UV	No homogenate (control)	20•1	(1•5)	–
BuP	Homogenate	UV	1	4•4	(2•6)	0•001*
BuP	Homogenate	UV	2	4•8	(0•4)	0•001*
BuP	Homogenate	UV	3	3•2	(0•7)	0•001*
BuP	Homogenate	UV	4	6•3	(3•4)	0•001*
BuP	Homogenate	UV	5	6•3	(1•9)	0•001*
BuP	Homogenate	UV	6	10•7	(3•2)	0•002*
BuP	Homogenate	UV	7	12•2	(4•3)	0•01*
BuP	Homogenate	UV	8	5•7	(1•1)	0•001*
BuP	Homogenate	UV	9	4•3	(2•9)	0•001*
BuP	Homogenate	UV	10	14•1	(3•0)	0•01*
BuP	Homogenate	Autoclaving	No homogenate (control)	3•4	(2•4)	–
BuP	Homogenate	Autoclaving	1	4•0	(1•1)	n.s.
BuP	Homogenate	Autoclaving	2	2•1	(1•7)	n.s.
BuP	Homogenate	Autoclaving	3	3•9	(1•4)	n.s.
BuP	Homogenate	Autoclaving	4	4•6	(1•2)	n.s.
BuP	Homogenate	Autoclaving	5	4•3	(1•7)	n.s.
BuP	Homogenate	Autoclaving	6	3•6	(0•7)	n.s.
BuP	Homogenate	Autoclaving	7	5•4	(2•1)	n.s.
BuP	Homogenate	Autoclaving	8	4•4	(1•7)	n.s.
BuP	Homogenate	Autoclaving	9	2•5	(1•6)	n.s.
BuP	Homogenate	Autoclaving	10	3•6	(1•5)	n.s.

* Inhibition

Table 56.

Growth of M13 in MLM with different Rf fractions after the fractionation of pine seedling root exudate in BuA. by paper chromatography.

Period of exudation	Method of sterilization of the fractions	Rf fraction	Mean dry weight (mg)	Standard error	P
3 weeks	UV	No exudate (control)	94•6	(6•7)	–
3 weeks	UV	1	99•0	(4•5)	n.s.
3 weeks	UV	2	103•7	(4•1)	0•05
3 weeks	UV	3	102•3	(11•2)	n.s.
3 weeks	UV	4	95•5	(7•4)	n.s.
3 weeks	UV	5	119•5	(28•1)	n.s.
3 weeks	UV	6	94•6	(15•3)	n.s.
3 weeks	UV	7	95•0	(1•7)	n.s.
3 weeks	UV	8	91•6	(7•2)	n.s.
3 weeks	UV	9	90•8	(9•1)	n.s.
3 weeks	UV	10	95•2	(4•6)	n.s.
3 weeks	Autoclaving	No exudate (control)	91•8	(18•6)	–
3 weeks	Autoclaving	1	95•3	(0•1)	n.s.
3 weeks	Autoclaving	2	100•4	(8•9)	n.s.
3 weeks	Autoclaving	3	103•3	(2•8)	n.s.
3 weeks	Autoclaving	4	87•8	(8•0)	n.s.
3 weeks	Autoclaving	5	93•0	(11•3)	n.s.
3 weeks	Autoclaving	6	95•3	(12•6)	n.s.
3 weeks	Autoclaving	7	94•6	(8•8)	n.s.
3 weeks	Autoclaving	8	89•7	(8•6)	n.s.
3 weeks	Autoclaving	9	93•9	(10•9)	n.s.
3 weeks	Autoclaving	10	87•5	(0•5)	n.s.

Continued .....

Table 56. (cont.)

Period of exudation	Method of sterilization of the fractions	Rf fraction	Mean dry weight (mg)	Standard error	P
25 weeks	UV	No exudate (control)	96•7	(6•3)	-
25 weeks	UV	1	127•8	(17•3)	0•01
25 weeks	UV	2	103•8	(30•8)	n.s.
25 weeks	UV	3	119•2	(35•9)	n.s.
25 weeks	UV	4	89•9	(2•4)	n.s.
25 weeks	UV	5	119•1	(7•4)	0•01
25 weeks	UV	6	100•8	(19•9)	n.s.
25 weeks	UV	7	86•2	(22•0)	n.s.
25 weeks	UV	8	80•4	(19•8)	n.s.
25 weeks	UV	9	97•9	(22•6)	n.s.
25 weeks	UV	10	68•4	(2•3)	0•001*
25 weeks	Autoclaving	No exudate (control)	19•2	(3•1)	-
25 weeks	Autoclaving	1	19•0	(1•1)	n.s.
25 weeks	Autoclaving	2	23•5	(3•8)	n.s.
25 weeks	Autoclaving	3	21•6	(3•8)	n.s.
25 weeks	Autoclaving	4	20•1	(4•5)	n.s.
25 weeks	Autoclaving	5	21•2	(3•7)	n.s.
25 weeks	Autoclaving	6	21•8	(1•4)	n.s.
25 weeks	Autoclaving	7	19•2	(3•9)	n.s.
25 weeks	Autoclaving	8	20•4	(4•7)	n.s.
25 weeks	Autoclaving	9	19•8	(2•5)	n.s.
25 weeks	Autoclaving	10	23•6	(8•9)	n.s.

* Inhibition

Table 57.

Growth of Boletus variegatus 1 in MLM with different Rf fractions after the fractionation of pine seedling root exudate in BuA, by paper chromatography.

Period of exudation	Method of sterilization of the fractions	Rf fraction	Mean dry weight (mg)	Standard error	P
3 weeks	UV	No exudate (control)	2•9	(0•7)	-
3 weeks	UV	1	0•1	(0•1)	0•01*
3 weeks	UV	2	0•1	(0•0)	0•01*
3 weeks	UV	3	0•0	(0•0)	0•01*
3 weeks	UV	4	0•3	(0•3)	0•01*
3 weeks	UV	5	0•5	(0•5)	0•02*
3 weeks	UV	6	1•4	(1•1)	n.s.
3 weeks	UV	7	0•1	(0•0)	0•01*
3 weeks	UV	8	0•1	(0•0)	0•01*
3 weeks	UV	9	1•5	(1•5)	n.s.
3 weeks	UV	10	1•1	(0•3)	0•05*
3 weeks	Autoclaving	No exudate (control)	5•0	(3•2)	-
3 weeks	Autoclaving	1	1•5	(1•5)	n.s.
3 weeks	Autoclaving	2	5•0	(2•9)	n.s.
3 weeks	Autoclaving	3	2•6	(0•1)	n.s.
3 weeks	Autoclaving	4	4•9	(1•6)	n.s.
3 weeks	Autoclaving	5	3•5	(2•0)	n.s.
3 weeks	Autoclaving	6	3•9	(1•5)	n.s.
3 weeks	Autoclaving	7	4•2	(0•3)	n.s.
3 weeks	Autoclaving	8	2•7	(1•6)	n.s.
3 weeks	Autoclaving	9	3•1	(1•1)	n.s.
3 weeks	Autoclaving	10	5•9	(1•0)	n.s.

Continued .....

Table 57. (cont.)

Period of exudation	Method of sterilization of the fractions	Rf fraction	Mean dry weight (mg)	Standard error	P
25 weeks	UV	No exudate (control)	14•2	(8•7)	-
25 weeks	UV	1	4•0	(3•5)	0•05*
25 weeks	UV	2	16•4	(9•5)	n.s.
25 weeks	UV	3	10•8	(4•7)	n.s.
25 weeks	UV	4	6•3	(1•0)	n.s.
25 weeks	UV	5	5•4	(5•1)	n.s.
25 weeks	UV	6	8•1	(2•3)	n.s.
25 weeks	UV	7	7•9	(3•9)	n.s.
25 weeks	UV	8	2•6	(1•6)	0•05*
25 weeks	UV	9	3•2	(0•7)	0•05*
25 weeks	UV	10	2•9	(0•5)	0•05*
25 weeks	Autoclaving	No exudate (control)	10•0	(7•4)	-
25 weeks	Autoclaving	1	9•4	(5•9)	n.s.
25 weeks	Autoclaving	2	12•2	(5•3)	n.s.
25 weeks	Autoclaving	3	12•5	(3•0)	n.s.
25 weeks	Autoclaving	4	10•7	(3•1)	n.s.
25 weeks	Autoclaving	5	13•5	(5•4)	n.s.
25 weeks	Autoclaving	6	13•4	(7•7)	n.s.
25 weeks	Autoclaving	7	14•5	(5•9)	n.s.
25 weeks	Autoclaving	8	9•5	(9•3)	n.s.
25 weeks	Autoclaving	9	5•9	(2•3)	n.s.
25 weeks	Autoclaving	10	15•9	(15•9)	n.s.

* Inhibition