The effect of herbicides on ectotrophic mycorrhizas

by

John Robert Smith

A thesis presented in part fulfilment of the requirements for the degree of Doctor of Philosophy of the University of London

1973

Botany Department,

Bedford College.

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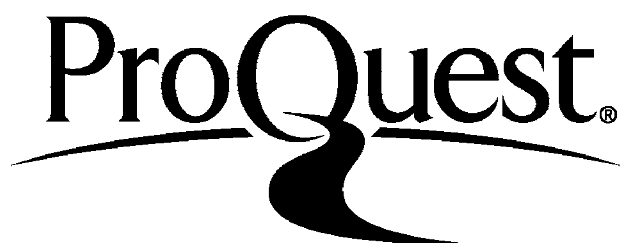

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ABSTRACT

The form and seasonal development of <u>in vivo</u> mycorrhizas of <u>P.sylvestris</u> seedlings growing in forest nursery plots 458FY70 (Kennington) and 83FY70 (Wareham) have been characterised; this enabled various characters of <u>P.sylvestris</u> seedlings (for example non mycorrhizal short roots, mycorrhizal roots, 'pioneer' and 'mother' and subordinate 'mother' roots) to be compared for different soil conditions.

Examination of the heterorhyzic root systems of <u>P.sylvestris</u> and <u>P.nigra</u> showed that long roots, for example 'pioneer' roots, are possibly more capable of adapting to a variety of soil conditions.

The effects of Simazine on measured aspects of the root systems of nursery grown <u>P.sylvestris</u> and <u>P.nigra</u> are unclear; equivalent Pines growing in the same soils contained in plant pots showed a demonstrable increase in both numbers of short roots and in mycorrhizas on the application of Simazine equivalent to 4 lbs Simazine per acre.

Growth measurements of known mycorrhizal and root pathogenic fungal isolates, on agar plates with added herbicides, showed that increase in growth of some isolates occurred in the presence of higher concentrations of Simazine or Atrazine. In contrast, equivalent experiments using Dichlobenil, Dichlobenzoate, Chlorthiamid and 245T indicated that identical isolates, growing in the presence of these herbicides, were inhibited.

These observations of herbicidal activity on mycorrhizas and their mycobionts were confirmed by measurement of respiration rates for *Boletus elegans* and *Amanita rubescens*, both mycobionts (the latter in very exceptional circumstances), and mycorrhizal roots of *P. sylvestris*. The extent of phosphate uptake by *P. sylvestris* mycorrhizas, estimated from buffered phosphate solutions containing herbicide, was also in agreement with the results.

Using a number of established techniques, attempts to produce reliable numbers of *in vitro* mycorrhizas were of limited success.

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(Full chemical names of biocides mentioned in text)

INTRODUCTION

Mycorrhizas were first defined and named by Frank in 1885. Since this time such mycorrhizas have been studied in some detail (Hatch, 1937; Kelley, 1931, 1950; Harley, 1940, 1948, 1952, 1969, 1971; Robertson 1954; Trappe, 1962; Wilde and Lafond, 1967; Peyronel et al, 1969) and are now known to be the result of an association involving the external tissues of the host root tips. Mycorrhizal fungi influence the function and development of tree roots, forming associations known as ectotrophic mycorrhizas, and differ from other root infecting fungi in that they rarely enter into a parasitic relationship with the host roots. Both Garrett (1950) and Harley (1948) regard mycorrhizal fungi as specialised, and evolved from saprophytic root-surface fungi.

Mycorrhizas are widely distributed in forest soils and involve many species of fungi. The varying specificity of mycorrhizal fungi for their hosts is not understood, although regular association of trees with particular fungal fruiting bodies (mainly basidiomycetes) has led to the publication of lists reporting probable ectotrophic mycorrhizal associations (Peyronel, 1921, 1922; Melin, 1936; Modess, 1941; Trappe, 1962). For example, Cenococcum graniforme, a widely distributed mycorrhizal fungus, forms dark-sheathed mycorrhizas with radiating hyphae on the root systems of many coniferous species (Trappe, 1962); Boletus elegans however, is only known to associate with Larix spp. (Melin, 1921; Trappe, 1962). Within these extremes lie the rest of the mycorrhizal fungi. In addition most tree hosts seem to be capable of forming mycorrhizal associations with several fungi (Melin, 1963; Trappe, 1964).

1. MORPHOLOGY, ANATOMY AND CLASSIFICATION OF MYCORRHIZAS.

Since the early studies by Frank in 1885, the gross morphology and anatomy of mycorrhizas has been studied in some detail. More recently, there have been partially successful attempts to classify mycorrhizas using these criteria (see below). Many of these studies of the morphology and anatomy of mycorrhizas concern mycorrhizas found on beech roots (Harley, 1937, 1940; Clowes, 1949, 1950, 1951, 1954). In addition Slankis (1948, 1949, 1958), MacDougal and Dufrenoy (1943, 1944), Scannerini(1968), Vozzo and Hacskaylo (1964), Hacskaylo and Vozzo (1965), Wilcox (1968) and other workers have studied the root systems of members of the Pinaceae. The mycorrhizal roots of the Pinaceae are commonly racemosly branched, but pinnate and monopodial root systems may also occur (McMinn, 1963; Bogar and Smith, 1965). Bjorkman (1942) has suggested that the particular morphology of ectotrophic mycorrhizas is a consequence of specific fungal infections, but this view is not generally held.

The size of the mycorrhizal fungal sheath and the depth of penetration of host root tissue by the mycorrhizal fungus is dependent on environmental conditions. This sheath of hyphae, both forming and surrounding the mycorrhizal roots, may be white, yellow, pink, red or black in colour (Moore, 1922; McArdle, 1932; Laing,1932; Kozlowski, 1949; Trappe, 1964; Wilcox, 1969). At high moisture levels white-coloured mycorrhizas seem to develop and to predominate; when soil moisture levels are lower they are replaced by black mycorrhizas. These black mycorrhizas are often abundant near the soil surface and to approximately six inches soil depth in dry sandy soil. This

distribution is believed to reflect drough resistance. Black mycorrhizas are easily confused with pseudo-mycorrhizal roots which are the result of infection of dead cortical cells by *Mycelium radicis atrovirens*, and the consequent pseudo-mycorrhizal roots appear as black, monopodial, mycorrhizal like roots (Melin, 1927; Laing, 1932; Robertson, 1954; Levisohn, 1954).

Anatomically, ectotrophic mycorrhizas consist of a core of host tissue surrounded by a mantle of fungal tissue. This mantle or sheath consists of two layers; an inner layer of hyphae intercellularly penetrating the host (sometimes as far as the endodermis) and an outer layer from which hyphae extend into the soil.

Dominik (1955, 1959) has proposed a form of classification of various external mycorrhizal associations, based on morphological characteristics. He takes as the basis for his classification any distinctive characters of hyphal and sheath structure. His classification implies that a specific mycorrhizal morphological type generally relates to only one fungus. These morphological types may belong, according to Dominik's scheme, to any one of several different primary groups and sub-groups. Dominik further observed that a mycorrhizal type could pass through several of these primary groups and through sub-groups (including colour sub-groups). Presumably these changes between groups and sub-groups depend on seasonal and other variations in both host root physiology and soil characteristics.

Trappe (1970) has proposed a classification scheme based on criteria similar to those of Dominik but with a different weighting. He lists the most stable characteristics of mycorrhizas as hyphal features rather than gross morphological features. Such features are: the presence and morphology of septal pores; the hyphal diameter and cell length; hyphal wall thickness; deposits of material on the hyphal walls; hyphal fluorescence and reaction to specific reagents. Further characters listed by Trappe as useful for categorising fungal symbionts are odour, taste, mantle structure, and colour.

Catalfomo and Trappe (1960) suggested a classification of mycorrhizas based on their phytochemistry. Krupa and Fries (1971) have since used gas spectrophotometry to discriminate between the mycorrhizal roots of P. sylvestris infected with different Boletus species.

Where the continuing relationship between a host and a mycorrhizal fungus is to be investigated, there is a need for the precise identification of the fungus. Correct taxonomic identification of fungi involved in mycorrhizal associations is not feasible using Trappe's, Dominik's or any other scheme. It seems doubtful whether any scheme could adequately separate the effects of different mycorrhizal fungi (on the morphology of ectotrophic mycorrhizas) from the effects of season, host and soil environment.

2. SOURCES OF MYCORRHIZAL INFECTION.

The existence of viable mycorrhizal propagules, perhaps including basidiospores of mycorrhizal fungi in the soil, is vital to the establishment of ectotrophic mycorrhizas. Robertson (1954) and Laiho and Mikola (1964) have established that basidiospores surviving in soils can account for the ectotrophic mycorrhizal infection of the long roots of seedling pines. In addition, there are reports of successful mycorrhizal syntheses using basidiospores, or mycelial suspensions, in association with peat moss, leaf litter or grain (Hatch, 1937; Rayner, 1938; McComb, 1943; Gilmore, 1968; Harley, 1969; Briscoe, 1959; Bowen, 1965, 1966; Park, 1969).

Mycorrhizal infection may also arise from rhizomorphs and hyphal strands of mycorrhizal fungi existing in the soil. Such structures may remain viable in the soil for many years after the decline of the host. Rayner and Neilson Jones (1944) reported the occurrence of Boletus bovinus rhizomorphs in almost treeless heathland.

It may also be possible for mycorrhizal fungi to occur as root fungal saprophytes. Romell (1938) has demonstrated that Boletus subtomentosus survived as such a saprophyte and formed fruiting bodies when the roots of the host, Pinus montana, were isolated by trenching. Dominik (1956, 1961) has reported the formation of a mycorrhizal association between Pinus montana and Fomes annosus, normally a virulent parasite. Boullard (1961, 1962) has shown a similar relationship between Armillarea mellea and Sitka

spruce. *Cenococcum graniforme* (probably a sterile ascomycete) is widespread both in soils previously bearing no known mycorrhizal host and in soils previously bearing suitable hosts. The fungus can readily form ectotrophic mycorrhizas with almost any host tree from mycelial or sclerotial propagules.

Once mycorrhizas have been established, whatever their source of infection, there is a simple progressive spread of mycorrhizal infection to other uninfected roots, and to uninfected trees (Mitchell, 1939; Roeloffs, 1930). In their first year of growth in new nursery soils conifers usually form unbranched mycorrhizas. Simple, non-dichotomous, mycorrhizal roots are more commonly found on trees growing in ground previously bearing conifers. In their first year of growth in any soil, nursery pines have small mycorrhizal roots which are either unbranched or simply forked. In their second year these pine mycorrhizas become branched; additionally, they grow in three or four seasonal phases throughout the year. Subsequent development of the mycorrhizal roots is related to this seasonal pattern of growth. The predominance of mycorrhizal fungi in the root-growing season may be due to the preferential stimulation of mycorrhizal fungi by root exudates. Seasonal decline and variation of mycorrhizal roots may be caused by the replacement of mycorrhizas with other root infections (Robertson, 1954; Wilcox, 1968; Krupa and Fries, 1971).

3. ISOLATION OF MYCORRHIZAL FUNGI AND THE SYNTHESIS OF MYCORRHIZAS IN VITRO.

About one hundred of these mycorrhizal associations have been confirmed by the pure culture technique of Melin (1936) which is the accepted means of establishing whether a fungus forms mycorrhizas. In this technique a mycorrhiza is synthesised *in vitro* between an aseptically grown seedling and a pure culture of the fungus. The mycorrhizal anatomy is confirmed by microscopic examination of sections cut from these mycorrhizal roots (Melin, 1936; Hacskaylo, 1953; Trappe, 1962).

Several early attempts were made to isolate the fungal associates from mycorrhizas; Müller (1903) and Peklo (1913) only isolated rhizosphere organisms; more recently workers have successfully isolated mycorrhizal fungi from mycorrhizal roots (Harley and Waid, 1955; Waid, 1957; Arnold, 1959; Zak and Bryan, 1963; Zak and Marx, 1964).

Fungal cultures derived from the sporocarps of basidiomycetes, fruiting in stands of trees whose roots bear ectotrophic mycorrhizas, are readily identifiable and as such have been useful for research work (Melin, 1936; Hacskaylo, 1953; Zak, 1969; Ferry, 1964). However the question of other basidiomycete mycelia parasitising the sporocarp of these fungi then arises (Watling, 1964, 1971; Griffith and Barnett, 1967).

Various results perhaps suggest that the mycorrhizal relationship consists of more than the 'simple' symbiosis of two organisms; Mikola (1965), Laiho (1967), and Vozzo (1969), have shown that nursery seedlings of coniferous trees inoculated with pure cultures of ectendotrophic mycorrhizal fungi do not respond as well as those receiving a soil inoculum. Similarly Mosse (1962) using an inoculum of an Endogone species failed to produce endotrophic mycorrhizas in the vascular tissue of strawberry plants, but succeeded when she introduced a third organism, a Pseudomonas species, into the system.

Both Zak and Marx (1964) and Lamb and Richards (1970) have shown that it is rarely possible to identify fungal isolates taken from tree mycorrhizal roots using, as a comparison, isolates from the sporophores of basidiomycetes regularly fruiting near the host tree. Both groups of workers have concluded that fungal species active in forming ectotrophic mycorrhizas are more numerous than has been reported; they also conclude that basidiomycetes commonly tested as mycorrhizal associates often have a minor role in mycorrhizal formation in the field. For example, Lamb and Richards (1970) could isolate neither Rhizopogon roseolus nor Boletus granulatus from the mycorrhizal roots of Pinus radiata, despite the fact that fruiting bodies of these fungi were present. Present reports of mycorrhizal associates must be treated with reservation; for example Trappe (1962) lists Rhizopogon roseolus and Boletus granulatus, with sixteen other fungi as mycorrhizal; his main

evidence is the occurrence of sporophores of these fungi in association with Pinus radiata.

Obviously techniques which can positively identify the fungal associates isolated from mycorrhizal roots need to be developed. Dominik (1956, 1959) and Dominik and Boullard (1961) have developed field keys to assist in the identification of the mycorrhizal fungus from the morphology of the host mycorrhizal root. These keys are of limited value since morphological variation of ectotrophic mycorrhizas occurs within a small range of soil environment (Zak and Marx, 1964). In consequence, Zak (1969) has developed a technique to identify the mycorrhizal fungus from the attached sporophore employing ultra-violet fluorescence to trace the soil rhizomorphs and hyphae leading from the mycorrhizas to the surface sporophores.

In view of these observations, perhaps it is better in laboratory syntheses of ectotrophic mycorrhizas, to isolate fungi from mycorrhizal roots rather than from sporophores. There are problems concerning the identification of the mycorrhizal fungus isolated from mycorrhizal roots, but the chances of successful mycorrhizal syntheses using mycorrhizal root isolates should be higher. In addition, the artifical formation of ectotrophic mycorrhizas, using pure cultures originating from sporophores, does not necessarily reflect the regular ability of these fungi to form mycorrhizas under natural conditions.

4. SEASONAL DEVELOPMENT OF MYCORRHIZAL FUNGI ON PINE ROOTS.

The pine root system consists of long lateral roots, some of which branch frequently to form long and short root tips. Both latter types of root tip show recurrent seasonal cycles of growth and dormancy and these root tips are colonised by mycorrhizal and non-mycorrhizal fungi (Ladefoged, 1939; Aldrich Blake, 1930; Robertson, 1954; MacDougal and Dufrenoy, 1944a, 1944b; Kramer, 1949; Orlov, 1957; Wilcox, 1964, 1968a, 1968b). Mycorrhizal fungi seem to predominate during the (root) growing season (Wilcox, 1968b). Non-mycorrhizal fungi may become dominant during the dormant season (Katznelson *et al* 1962). Robertson (1954) has noted that there is an inverse relationship between *M. radicis atrovirens* behaving as a facultative parasite on the tips and bases of the long roots of *P. sylvestris* and the mycorrhizal infection of the short root tips. He attributes this relationship to the early death and collapse of the cortex of the long roots which do not develop into mycorrhizas, and consequently form a substrate for the parasitic fungus.

Following dormancy, reactivation of the meristematic tissue of the long lateral roots and the long and short root tips occurs. Since there is a lag between the onset of root growth and subsequent mycorrhizal infection, mycorrhizal fungi would seem to be stimulated by meristematic reactivation of root tissue rather than *vice versa* (Wilcox, 1954, 1964, 1967, 1968a).

Robertson (1954) has shown that in Scot's Pine, long lateral roots are normally infected by a Hartig net. Short roots are commonly mycorrhizal, the source of infection being the Hartig net which extends along the long lateral root. Hyphal growth from the Hartig net is stimulated by the reactivation of the long lateral root, and even a delay in the growth of the fungus appears insufficient to permit emerging short root laterals to escape infection from the Hartig net. Only first order laterals and large'pioneer' roots can grow sufficiently rapidly to escape infection by the Hartig net. When this occurs new lateral roots become fungal-free, but may later become infected from another source.

The mycorrhizal development of Scot's Pine seedlings occurs three to four weeks after the formation of the first short roots (Laiho and Mikola, 1964). These authors have noted that mycorrhizal development of Scot's Pine seedlings continues steadily throughout the growing season, with up to 60% of the short roots becoming infected by the end of the first growing season, and up to 90% by the end of the second growing season.

Ladefoged (1939) has also noted a relationship between mycorrhizal activity and the growth of short roots in Norway spruce. In this instance the relationship between short root growth and mycorrhizal activity occurred from mid June to mid July; this period of growth was followed by a decline of mycorrhizal activity and short root growth. Strong development of short roots and mycorrhizal activity recommenced in August. This activity lasted until mid

August and was followed by a further decline.

Conditions of soil nutrient deficiency or unbalance seem to favour the development of mycorrhizas (Melin, 1917; Kessell, 1927; Roelloffs, 1930; Cheyney , 1932; Hatch, 1937; Kramer and Kozlowski, 1960; Rayner, 1938; Dimbleby, 1953). In these conditions of soil nutrient deficiency, the carbohydrate status of the whole plant is likely to be high, but this is perhaps incidental to mycorrhizal formation (Harley, 1969). The carbohydrate status of the tree root may vary, depending on the dormancy or activity of the root meristems, and this may explain the appearance, the disappearance, and the varying activity of both mycorrhizal and non-mycorrhizal fungi on the host root surface.

5. THE FUNGAL INFLUENCE.

In the early stages of mycorrhizal root development the extent to which the fungus influences the gross morphology and the anatomy of the mycorrhizas is not clear. However, later morphological development, i.e. regular dichotomous branching of the mycorrhizas, is clearly influenced by the fungus (Warren Wilson, 1951).

In a review in 1958, Slankis reported that a root morphology closely resembling that of mycorrhizal roots can be obtained with externally applied auxin. He noted however, that non-mycorrhizal roots of Scot's Pine may become dichotomous in the absence of externally applied auxin. He believes (1948, 1949, 1950, 1951, 1967) that the particular morphology of mycorrhizal roots is due to a state of hyperauxiny caused by the production of auxin by the mycorrhizal fungus. The observations of Moser (1959), Ulrich (1960) and Hanak (1964) support this view. A number of mycorrhizal fungi are known to be capable of synthesising indole-acetic acid from tryptophan, and Levisohn (1960) has noted that tryptophan (a known precursor of indole-acetic-acid) caused root forking in pines. However, Ritter (1968) showed that enzyme preparations from pine and birch roots which normally caused indole-acetic acid destruction (and therefore regulate levels of indole-acetic acid in root tissues) were inhibited by culture solutions of *Boletus bovinus*. Inhibition of these enzymes would cause hyperauxiny in mycorrhizal roots and induce changes in the root morphology; this would give rise to the root structures peculiar to ectotrophic mycorrhizal roots.

6. THE HOST EFFECT.

Most workers have assumed that anatomical and gross morphological differences between the host tissues of mycorrhizal and uninfected roots relate to fungal activity. However Warren-Wilson (1951) has found evidence of ageing in the root apices in uninfected beech root tips, and suggests that these branch rootlets growing in natural soils may become modified before mycorrhizal infection. In addition, in some circumstances, the effect of the tree host is very obvious. For example, the gross morphology of mycorrhizas developing on infected pine and beech roots are basically different. Infected pine roots produce dichotomous mycorrhizas whilst beech roots produce simply branched mycorrhizas.

Harley (1969) suggested that two morphogenetic changes occur in established mycorrhizal roots. The host root itself undergoes an ageing process (see above) and concurrently there is a maintenance of growth and branching due to fungal activity. It may be that certain chemical products implicated in mycorrhizal formation in vitro, e.g. the M-factor (Melin, 1954, 1959) do not necessarily affect the morphogenetic development of mycorrhizas.

7. CARBON NUTRITION.

Clearly, in order to function, the mycobiont requires a supply of carbon and energy. Bjorkman (1942) and others have claimed that the development and maintenance of mycorrhizas depends on a supply of carbohydrates in the roots of the tree and the general consensus of opinion seems to be that this is so. Foster and Marks (1966) have shown that cells of the root cortex of *Pinus radiata*, uninfected by the mycorrhizal fungus, contain starch filled amyloplasts; cortical cells in contact with the mycorrhizal fungus of the Hartig net contain empty amyloplasts. Most known mycorrhizal fungi lack cellulase (Melin, 1946) and seemingly need to obtain carbohydrates from the host roots. However, certain mycorrhizal fungi are exceptional in that they are able to obtain their own carbohydrate supply from the breakdown of insoluble organic matter (Young, 1940). MacDougal and Dufrenoy (1944, 1946) isolated mycorrhizal pine roots symbiotic with *Elaphomyces* species and maintained these isolated roots in a medium containing organic carbon for 28 months. They suggest that some of the root wood synthesised may be from the carbohydrate produced by the mycorrhizas; an alternative explanation might be that the root grew as an excised structure and supported the fungus. Soil carbon dioxide may also contribute to the carbon supplies of mycorrhizas *in vivo* (Brierly, 1955). Harley (1964) has demonstrated that beech mycorrhizas assimilate labelled bicarbonate ions into labelled intermediates.

Carbon, in the form of various carbohydrates obtained from the host roots, is converted by the mycorrhizal fungal sheath

into forms which cannot be utilised by the host. Lewis and Harley (1965) found that 55-75% of stump applied ^{14}C sucrose passing into root tips was found in the fungal sheath as trehalose, mannitol, and glycogen.

Tranquillini (1959, 1964) has calculated that 40% of the photosynthate translocated to the roots of beech seedlings would be used by mycorrhizal fungi. Harley and McCready (1965) and Harley (1969) note that the fungal sheath contains 39% of the dry weight of excised mycorrhizal roots of beech.

8. FUNCTIONING OF MYCORRHIZAS AS ORGANS OF ABSORPTION.

There have been many reports (see reviews by Hatch, 1937; Rayner, 1927; Rayner and Neilson Jones, 1944; Levisohn, 1958; Hackskaylo, 1969; Harley, 1971 and Fortin and Pineau, 1971) of increased growth of trees whose roots are infected by mycorrhizal fungi, compared with trees whose roots are not mycorrhizal. Many of these reports of increased growth concern trees infected with mycorrhizal fungi developing in infertile soils or in soils lacking one or more major elements. Parallelling these observations of increased tree growth there are reports of increased nutrient uptake in such infected trees. Conditions of optimum or excess soil nutrition do not necessarily prevent mycorrhizal development but usually reduce the intensity of mycorrhizal infection (Rosendahl, 1943; Stone, 1950; Stone and McAuliffe, 1954; Linneman and Meyer, 1958; Richards, 1961, 1965). This increased growth of trees bearing mycorrhizal roots is associated with an increase in dry weight and an increase in both root tips and branches compared with trees that do not possess mycorrhizas (Hatch, 1937; Morrisson, 1961; Wakeley, 1965; Rayner, 1939; Rayner and Levisohn, 1941; Rayner and Neilson Jones, 1944; White, 1941; Finn, 1942; Rosendahl, 1943).

Hatch (1937) has pointed out that the surface area of the tree root tips increases when the tips become mycorrhizal; he also indicates that the hyphae radiating from the mantle into the soil will increase the absorbing area. Hatch implies from this that the benefit of mycorrhizas to the host arises directly from an increase in the host root absorbing area. However, there would seem to be

no advantage in replacing host root hairs, which are abundantly developed in fertile soils, with mycorrhizal fungal extensions.

Melin et al (1950, 1952, 1953a, 1953b, 1955, 1958) have established that hyphae actively absorb and translocate nutrients from synthetic media to the host, via mycorrhizal roots. Voight (1969) has suggested that mycorrhizas initiate most of the nutrient cycles in infertile soils. These soils (defined by Voight) are characterised by the lack of one or more of the soil nutrients absorbed by either uninfected root tips or by mycorrhizal roots (and their soil hyphal extensions). Voight also accords the presence of mycorrhizas to the lack of organic or inorganic colloids in these infertile soils. Other work has shown that the mycelia radiating from the mantle of mycorrhizal roots can solubilise the soil silicates contained in the soil particles, absorb the released ions and transport these ions to the tree root system (Rosendahl, 1943; Wilde and Iyer, 1962; Henderson and Duff, 1963; Voight et al, 1964; Voight, 1969).

On a per plant basis mycorrhizal roots contain twice as much nitrogen and potassium, and four times as much phosphorus,as non-mycorrhizal roots. When these same results are expressed on a unit dry weight basis, the same mycorrhizal roots can contain twice as much phosphorus as non-mycorrhizal plants, but there is only a small difference in the plant contents of nitrogen and potassium (McComb, 1938, 1943; McComb and Griffith, 1946; Mitchell, 1939; Kramer and Wilbur, 1949; Harley and McCready, 1950; Morrisson, 1961). Harley and McCready (1950) have shown experimentally that excised

roots of beech absorb labelled phosphate more efficiently than uninfected roots.

In certain soil conditions relating to seasonal changes, uptake of phosphate from the soil decreases and phosphate within the fungus is mobilised and enters the host (Kramer and Wilbur, 1949; Harley et al, 1950, 1952a, 1952b, 1953, 1954, 1955, 1958; Clode, 1956; Lobanow, 1960; Bowen and Theodorou, 1967). In addition there is some indirect evidence that mycorrhizal roots somehow stimulate the accumulation of nitrogen in plants.

9. THE PROTECTIVE FUNCTION OF MYCORRHIZAS.

(a) ANTIBIOTICS.

In pure culture many ectotrophic mycorrhizas produce antibiotics active against root pathogenic fungi and bacteria, but these antibiotics have not so far been demonstrated to be functional biological control mechanisms under natural conditions. On the other hand, mycorrhizal fungi may be directly influenced by antibiotics produced by soil actinomycetes (Muller, 1960) and by soil fungi (Rayner and Neilson Jones, 1944; Levisohn, 1952).

Ectotrophic mycorrhizal roots may be less susceptible to infection by root pathogens than non-mycorrhizal roots. Zak (1964) has postulated that mycorrhizal fungi may protect root tips from infection by fungal pathogens in a number of ways; firstly, by utilising root carbohydrates and other exudates and reducing the 'attractiveness' of the root to pathogens; secondly, by providing a mechanical barrier to the pathogens in the form of a fungal mantle; thirdly, by producing antibiotics which may inhibit or kill the potential pathogens; and fourthly, by attracting a 'protective' rhizosphere population around a mycorrhizal root system.

There is some evidence for Zak's postulates; for example, Rypacek (1960) has shown that cultures of Boletus variegatus were antagonistic towards some parasitic wood destroying hyphomycetes, Phellinus hartigii, Paxillus atromentarius and Fomes marginatus, but not towards some saprophytic wood-decomposing fungi, Osmoporus oderatus, Schizophyllum commune and Xylaria hypoxylon. In addition,

Sasek (1967) and Sasek *et al* (1968) demonstrated antibiotic activity in a variety of mycorrhizal basidiomycetes against *Rhizoctonia solani*; similarly Hyppel (1968) noted that the *in vitro* growth of *Fomes annosus* was inhibited by 40 of the 85 mycorrhizal fungus isolates tested. Hyppel cites isolates of *Boletus bovinus* and *Boletus variegatus* as the most antagonistic to the growth of *Fomes annosus*. Santoro and Casida (1962) established that acetone extracts of *Boletus luteus* were active against gram positive bacteria. Histological evidence for Short Leaf and Lob lolly Pine mycorrhizas with intact mantle and Hartig net showed that both Pines were resistant to infection from zoospores of *P. cinnamomi* (Marx and Davey, 1969, a and b).

Santoro, Grand and Casida (1964) found that an antibiotic produced by *Gnococcum graniforme* was active against bacteria, yeasts, and actinomycetes. Production of antibiotics by mycorrhizal fungi may affect their hosts directly by altering cell wall metabolism or cell permeability or indirectly by preventing pathogenic infection (Santoro and Casida, 1962; Zak, 1964; Hypell, 1968; Marx, 1969, a and b). Marx and Davey, 1967, 1969a, 1969b; Marx and Bryan, 1969).

Recently Krupa and Fries (1971) have shown that *Boletus variegatus* produces ethanol, isobutanol, isoamyl alcohol, acetoin, and iso-butyric acid; both isobutanol and isobutyric acid are known to be generally fungistatic. Infection of *Pinus sylvestris* roots with *Boletus variegatus* results in the accumulation of volatile terpenes and sesquiterpenes in concentrations two to eight times greater than in uninfected *Pinus sylvestris* roots. Melin and Krupa (1971) found that these terpenes and sesquiterpenes were fungistatic.

Vapour from the terpenes and sesquiterpenes inhibited growth of Boletus variegatus and Rhizopogon roseolus from 55% to 86%. The suggestion is that terpenes and sesquiterpenes restrict the growth of the mycorrhizal fungi within the host roots, whilst producing a resistance of mycorrhizal root systems to root pathogens.

9. THE PROTECTIVE FUNCTION OF MYCORRHIZAS.

(b) EXUDATES.

Despite extensive evidence for the exudation of organic material from roots, and the probable role of such compounds in setting up and maintaining a rhizosphere population and mycorrhizas, there is little evidence of any specific effect of root exudates on a normal microflora. The volume of soil around the root into which exudates diffuse, and the extent to which exudates are adsorbed by soil colloids, is unknown. Schroth and Snyder (1960) have demonstrated that the root tip is the most important zone of root exudation. Katznelson, Rouatt and Peterson (1962) have found that the rhizosphere of Yellow Birch mycorrhizal roots contained a different population of fungi, and a larger population of bacteria, than the rhizosphere of non-mycorrhizal roots. These different rhizosphere populations are likely to reflect the presence or absence of potential mycorrhizal fungi.

Chromatographic analyses performed on root exudates indicate that they contain vitamins, organic acids, carbohydrates and many other compounds (Melin and Das, 1954; Rovira, 1956; Krupa and Fries, 1971). Frenzel (1960) used specific mutants of *Neurospora* to show that threonine and asparagine exude from the root tip of *Helianthus annuus*, whilst leucine, valine, and phenylalanine exude in greater amounts from the root hair zone. In addition Rovira (1965) reviews the work of many researchers and lists the amino acids exuded from different parts of various plant roots. With regard to the effects of environmental patterns Rovira (1959) has

shown that high light intensity increased amino acid exudation in plant roots. Slankis (1958) demonstrated the appearance of photosynthetically fixed ^{14}C in many of the compounds which form the root exudates of Pinus strobus; malonic acid was the major radioactive exudate.

There is much evidence that root exudates contain substances which are essential to the vigour of mycorrhizal fungi. Melin demonstrated the existence of substances stimulating the growth of mycorrhizal fungi in the exudates of excised pine roots (1925), in yeast extract (1939), and in fresh leaf litter (1946). Later (1954) he showed that the growth of both Boletus variegatus and Boletus elegans was increased in the presence of tomato, wheat, hemp, or pine roots. He termed the factors causing this stimulation the 'M' factor or stimulating principle. Melin also demonstrated that some mycorrhizal fungi, for example Russula spp, Lactarius spp and Cortinarius pholiota, were completely dependent on the presence of the 'M' factor for their growth in culture. He found that low concentrations of 'M' factor stimulated Boletus variegatus, whilst high concentrations inhibited growth of the fungus. He suggested that inhibiting and stimulating principles were obtained from different parts of the root system; the older roots producing inhibitory substances, whilst the younger primary rootlets produce stimulating principles.

10. THE BREAK-DOWN OF SYMBIOSIS.

Ectotrophic mycorrhizas on the roots of forest trees are generally accepted as necessary for the survival and growth of trees in acid, nutrient-deficient soils (Melin, 1917; Rayner, 1938; Dimbleby, 1953; Wright, 1957; Hacskaylo, 1972; Persidsky and Wilde, 1960; Park, 1971). Hatch (1937) found that the addition of balanced nutrients to soil resulted in poor mycorrhizal formation and concluded that the intensity of mycorrhizal formation was controlled by internal nutrient levels in host roots.

Under certain conditions the mycorrhizal fungi may behave parasitically, and this was shown by Melin (1922, 1923, 1927), who considered that vigorous mycorrhizal fungi may parasitise plant roots. Laing (1932) has reported the destruction of root tissues by mycorrhizal fungi, and the complete destruction of all tissues external to the endodermis, as common on peaty soils.

Septate hyphae of ectotrophic mycorrhizas of *Pinus radiata* were observed by MacDougal and Dufrenoy (1944b) to destroy the epidermal and hypodermal cells of the root, to traverse the middle lamella of the cortical cells, and to send hyphae into the vacuoles of these cells.

It can be seen from the above brief survey that the nature of the relationship between mycobiont and host is a finely balanced one. In fact Dominik (1961) and Boullard (1961, 1962) have noted the converse, that is the development of mycorrhizal roots as a result of infection by *Fomes annosus* and *Armillarea mellea*, both common root pathogenic fungi.

11. THE EFFECT OF SOIL STERILANTS, FUNGICIDES, INSECTICIDES AND HERBICIDES (BIOCIDES) ON MYCORRHIZAS.

Under certain conditions of nursery practice, unequal application of either soil sterilants, fungicides, insecticides or herbicides (biocides) from different soil treatments may lead to relatively high concentrations in the soil. These soil concentrations of active biocides may be higher than the annual applications.

High biocide concentrations in the soil may indirectly affect the inoculum potential of any mycorrhizal fungal propagules in the nursery soil; hence affecting the degree of mycorrhizal infection of nursery seedlings. High levels of biocides in the soil may also affect the distribution of root pathogenic fungi and thus indirectly affect the development and morphology of the nursery seedling roots.

The consequent ability of these nursery seedlings to survive under forest conditions is likely to be dependent on the effect of the nursery practice on the development of the mycorrhizal roots. Mycorrhizas are known to be protectants against soil pathogens, and any lag in mycorrhizal development is likely to impede the host root's immunity against root pathogens (Afshapaur 1967; Sobotka, 1968, 1970).

Soil sterilisation

In certain soil conditions (where the populations of root

pathogens may present a considerable problem), complete soil sterilisation effectively controls the spread of root disease. This complete soil sterilisation often causes a "biological vacuum", however, resulting in the long term absence of mycorrhizal fungi, and the relatively rapid reintroduction of root patho-genic fungi.

Hence in established nursery practice, there is a tendency towards the minimal use of soil sterilants, and towards the use of partial sterilants which do not completely sterilise the soil. For example, ethylene dibromide, carbon disulphide, formaldehyde, chloropicrin and methyl bromide can partially sterilise the soil, temporarily reduce the numbers of soil micro organisms and selectively destroy the most vulnerable root pathogenic and saprophytic fungi. Survivors of this partial soil sterilisation (which usually include *Trichoderma viride*) grow profusely and become antagonistic toward root pathogens such as *Rhizoctonia* spp. This rapid growth of the survivors is attributed to the increased energy being made available to the nitrogen fixing bacteria present in the soils; these bacteria consequently raise the levels of ammonia, nitrate, and amino acids present in the soils(Altman, 1963, 1964, 1970; Altman and Tsue, 1965).

Several workers have noted that root pathogenic fungi either become resistant to soil sterilant or, due to their inherent resistance, occupy the 'void' caused by the death of susceptible fungi. For example, a low application of chloropicrin may be followed by an attack of *Verticillium albo-atrum*, while soil applied PCNB inhbits

Rhizoctonia solani but not *Pythium* spp. Surface soil treatments of 'Vapam' protect the crop roots throughout their early growth, but *Pythium* spp. and *Phytophora* spp. may later infect tap roots which grow down to the lower layer of untreated soil and hence infect the whole root system (Baker, 1970). Consequently, soil sterilisation may result in the absence of soil fungi for comparatively short periods of time. The soil may be effectively sterilised to five cm. soil depth, but below this level root pathogenic fungi are active and can be rapidly reintroduced into the upper soil layers. Following such soil sterilisation a normal soil microbial population is eventually restored and root pathogens may again become prevalent (Brind, 1965).

Since mycorrhizal fungi are most common in the upper soil layers (Kreutzer, 1965), soil sterilisation often results in the long term absence of mycorrhizal fungi. Nowadays, because of the problem of rapid re-establishment of root pathogenic fungi, soil sterilants have been largely replaced by selective fungicides. Fungicides used for this purpose include PCNB, 'DD' and Dexon. These fungicides are both fungi-toxic and persistent in treated soils and they may not be as deleterious to the mycorrhizal fungus.

12. THE EFFECT OF SOIL APPLIED STERILANTS AND FUNGICIDES ON THE SURVIVAL OF NURSERY SEEDLINGS.

Levisohn (1965) noted that after partial soil sterilisation an improvement of tree seedling growth in nurseries bore no relationship to either the level of pathogenic root infection, or to the formation of mycorrhizas. Benzian (1968) found that stunted tree seedlings, growing on neutral soil, were improved by either acidifying the soil or by partially sterilising the soil with steam, formalin, chloropicrin, or chlorobromopropane. Benzian(1965), Ingestad and Molin (1960), and Will (1962) reported growth responses of conifer seedlings after partial soil sterilisation in nurseries where soil pathogens did not normally appear to affect tree growth.

Ram Reddy, Salt, and Last (1964), and Griffin (1965), could not find an adequate explanation for the stimulation of the growth of seedling conifers after partial soil sterilisation. They argued that if the stimulation of tree growth was related to the suppression of soil pathogens, then tree growth responses would differ with seasonal variations in the activity and growth of the soil pathogens. However, this seasonal variation has not been demonstrated. A better explanation has been put forward by Altman (1963, 1964, 1970), who suggested that partial soil sterilisation increases the activity of nitrogen fixing bacteria, consequently increasing the level of soil nitrate, soil ammonia and soil amino acids. These higher levels of soil nitrate, soil ammonia and soil amino acids are easily assimilated by the nursery seedlings, irrespective of any damage to their roots by soil pathogens. Consequently, healthy nursery seedlings can sometimes be grown on partially sterilised

soil containing no effective mycorrhizal fungi.

However, there are some reports that the elimination of mycorrhizal fungi by either soil sterilisation or soil applied fungicides may severely reduce the uptake of phosphorus and other essential elements from soils which possess these nutrients both in abundance and in readily absorbable forms (Henderson and Stone, 1967; Iyer Lipas and Chesters, 1969).

There is a report by Henderson and Stone (1967) that phosphorus deficient nursery seedlings though apparently healthy and showing vigorous growth may have such reduced root systems that the subsequent survival of these seedling trees when transplanted in to forest conditions may be significantly decreased. Similarly, Iyer, Lipas, and Chesters (1969) found that the prolonged use of 'Mylone' and 'Vapam' in nurseries bearing Red Pine seedlings increased the seedlings' nitrogen content, at the same time decreasing both potassium and phosphorus contents of the seedling foliage. Iyer, Lipas and Chesters found that the nursery seedling trees were characterised by their succulent shoots and small root systems.

13. THE EFFECT OF FUNGICIDES AND HERBICIDES ON MYCORRHIZAL FUNGI.

The effects of fungicides and herbicides are easily quantified by examining the shoot:root ratios, the development of the root system, the root surface area, the density and succulence of tissues (Iyer and Wilde, 1965; Iyer, 1964). An accurate assay of their effect on the soil mycorrhizal fungi can be made by assessing the degree of mycorrhizal infections of aseptic tree seedlings transplanted into soils treated with either fungicides or herbicides (Wilde, 1954; Persidsky and Wilde, 1960; Voight and Persidsky, 1956; Palmer and Hacskaylo, 1957).

Using the latter techniques it can be shown that soil applied fungicides such as allyl alcohol, formaldehyde, and methyl bromide, can apparently have a deleterious effect on the ectotrophic, rhizoplane, and rhizosphere fungi of forest tree roots (Wilde, Voight and Persidsky, 1956; Palmer and Hacskaylo, 1957; Persidsky and Wilde, 1960; Iyer and Wilde, 1965; Laiho and Mikola, 1965). Other soil applied fungicides seem to disrupt the mycorrhizal synthesis, either affecting the host tissues or mycorrhizal morphology (Simkauer and Shenefelt, 1952 Persidsky and Wilde, 1960; Nesheim and Linn, 1969).

Other soil applied fungicides, for example allyl alcohol and 'thiram' affect the coniferous root system rather than the mycorrhizal fungi. Such fungicides may alter the number of short roots and the root volume and this effect may result in the apparent decline in the number of mycorrhizal roots. This decline may be due

to physical changes in the root system rather than changes in the infectivity of the mycorrhizal fungi (Voight, 1955; Wilde, Voight and Persidsky, 1956; Persidsky and Wilde, 1960).

Some herbicides, especially those belonging to the triazine group, may indirectly affect the morphology of mycorrhizas, altering the frequency of different ectotrophic mycorrhizal fungi. For example, Uhlig (1966a, 1966b) observed that soil applied 'Simazine' stimulated growth of the mycorrhizal fungi *Tricholoma pessundatum* and *Scleroderma vulgare*. This stimulation, he considered, improved mycorrhizal formation on the host roots of *Picea abies* and *Pinus sylvestris*.

The present investigation was to investigate herbicide effects on mycorrhizas, mycorrhizal fungi, and root pathogenic fungi.

Mycorrhizal fungi are known to be beneficial to host tree roots; root pathogenic fungi are usually deleterious to the host. Attempts were made to see if herbicides caused any break-down in the mycorrhizal relationship.

Simazine, Atrazine, 245T, Diclobenil, Chlorthiamid, and 2,6,Dichlorobenzoic acid were studied to see if they affected the in vitro growth or the respiration rates of mycorrhizal and root pathogenic fungi. The same herbicides were also studied to see if they affected the respiration rates and the uptake of inorganic phosphate by mycorrhizal roots of P. sylvestris.

The agar plate and liquid culture experiments were designed to determine the concentration of any herbicide effect on the mycorrhizas of P. sylvestris.

Both the mycorrhizal and the root pathogenic fungi chosen for these investigations associated with either P. sylvestris or P. nigra. The vegetative mycelium of these mycorrhizal and root pathogenic fungi is probably most affected by herbicides. In order to ascertain the selective stimulation or inhibition of any of these isolates by herbicides, some of the work concerned the investigation of herbicide effects on pure cultures of mycorrhizal and root pathogenic fungi.

Reports in the literature suggest that Simazine and Atrazine may stimulate both the development and population of rhizosphere organisms.

A report that Simazine stimulated both the in vitro and the in vivo growth of Tricholoma pessundatum and Scleroderma vulgare (both mycorrhizal fungi), together with reports that Triazine herbicides increased the growth and the overall vigour of commercially grown crops, led to the assumption that these groups of herbicides might affect the mycorrhizal status of nursery grown pines.

MATERIALS AND METHODS.

1. THE FORM AND DEVELOPMENT OF PINE ROOT SYSTEMS.

The root systems of two year old nursery grown pines taken from Forestry Commission plots 458FY70 (Kennington, Oxfordshire) and 83FY70 (Wareham, Dorset) were carefully examined and compared with existing classification schemes for pine roots (Aldrich Blake, 1930; Wilcox, 1964; Sutton, 1969).

The heterorhyzic features of the pine root system, that is woody, long lateral roots and non woody, short roots, described by Aldrich Blake (1930) were identified.

Woody, long lateral roots were further classified into 'pioneer', 'mother' and 'subordinate mother' roots (Wilcox, 1964). 'Pioneer' roots were identified as roots with very short branches; 'mother' roots were smaller in diameter, shorter and plentifully branched; 'subordinate mother' roots were still smaller in diameter and more densely branched.

Short roots were commonly mycorrhizal; attention was paid to the assessment of the form and development of these mycorrhizal roots.

a) Assessment of the form and development of *Pinus* root systems.

The Pine root systems of the nursery seedlings sampled in 1970 were assessed as:

1. Total number of root tips
2. Total number of root tips that are mycorrhizal
3. Fresh weight of the root system
4. Dry weight of the root system.

The correlations between the four variates, together with standard deviations for each variate, were calculated. A standard computer programme developed by the University of Los Angeles Bio-Medical Department (classified as BMD 07M, Stepwise discriminant analysis) was used for the calculations. Part of the output from this programme consists of:

1. Group means and standard deviations.
2. Within groups covariance matrix.
3. Within groups correlation matrix.

To test the significance of the correlation coefficients obtained for the 1970 assessments, a very large sample of 465 *P. nigra* nursery seedlings was taken (in February 1972) from Wareham nursery, plot 83FY70. In large samples (in the region of 500 or above) the distribution of assessed values is symmetrical and usually distributed around a central average value; the correlation coefficient tends to have a normal distribution with the mean and the standard deviation. Seedlings growing in forest plot 83FY70 (Wareham), in soils containing either 0, 1, 2, 4, or 8 lbs Simazine/acre, give a correlation coefficient of 0.96258 ± 0.0034 between shoot fresh weight and shoot dry weight. It is safe to

assume that this correlation and some other very significant correlations obtained have a value of 1.0.

Other correlations for smaller samples of trees have similar values; for example the correlation coefficient of the root fresh weight and root dry weight of 254 P. sylvestris seedlings growing in forest plot 83FY70 (Wareham) is 0.9655 ± 0.0054 (Table 1).

Even in these smaller samples the distribution of the assessed variates must be normal, for all the correlations obtained are approximately the same as those calculated for the larger sample. Correlations between shoot fresh weight and shoot dry weight, and between root fresh weight and root dry weight, were high enough to assume that it was sufficient in subsequent experiments just to measure root and shoot fresh weights.

There is some correlation between the total number of non-mycorrhizal short roots and the fresh weight of the root; for example P. sylvestris (Wareham) 0.70446 ± 0.03 (254 sample size); P. nigra (Kennington) 0.78458 ± 0.0390 (232 sample size) (Tables 1 and 2). It seems reasonable to suppose that the numbers of non-mycorrhizal short root tips must increase with an increase in either root fresh weight or root dry weight. Hence the total number of short roots per whole root system might be estimated using a small sample of root system. However, other characters of the root system bear no relationship to any other characters, nor to themselves; for example the numbers of 'pioneer' and 'mother' roots (Wareham: -0.3377). Since there are very few, if any, root tips on 'pioneer' roots (Aldrich Blake, 1937; Wilcox, 1964) and there is no relationship

between 'mother' and 'pioneer' roots; the total number of root tips per plant cannot be extrapolated from the number of short roots per gram fresh weight root system (Table 2).

For these reasons all the pot experiments and the experiments sampled in February 1972 and November 1972 were assessed for the number of 'pioneer' roots, number of 'mother' roots (including 'subordinate mother' roots), as well as the fresh weight of the shoot, the fresh weight of the root, the number of dichotomous roots, the number of mycorrhizas, the number of lateral roots and the number of short roots.

Table 1. Some correlation coefficients obtained for seedlings of *P. sylvestris* and *P. nigra* growing on forest plots 458FY70 (Kennington), 83FY70 (Wareham), and in Thetford Forest.

Sample time	Seedling	Forest plot	Characters assessed	Correlation coefficient
Nov,70	*P. sylvestris*	Wareham	Root fresh weight/number of uninfected root tips	0.40179
Nov,72	"	"	"	0.70446
Nov,70	"	Kennington	"	0.44295
Nov,70	*P. nigra*	Wareham	"	0.54789
Feb,72	"	"	"	0.53528
Nov,70	"	Kennington	"	0.78458
Nov,70	*P. nigra*	Wareham	Root fresh weight/shoot fresh weight	0.75403
Nov,70	*P. sylvestris*	Wareham	"	0.96213
Nov,70	*P. nigra*	Wareham	Root fresh weight/root dry weight	0.86000
Feb,72	"	"	"	0.87718
Nov,70	"	Kennington	"	0.61900
Nov,70	*P. sylvestris*	Kennington	"	0.82010
Nov,71	"	Thetford	"	0.95102
Nov,70	"	Wareham	"	0.96550

Table 2. Some correlation coefficients obtained for seedlings of *P. sylvestris* and *P. nigra* growing in plant pots at Bedford College.

Sample time	Seedling	Soil type	Characters assessed	Correlation coefficient
Nov, 70	*P. nigra*	Wareham	Root fresh weight/number of uninfected root tips per gram	0.49802
Nov, 71	"	Thetford	"	0.70490
Nov, 70	"	Kennington	"	0.60293
Nov, 70	*P. sylvestris*	Wareham	"	0.56227
Nov, 71	"	Thetford	"	0.67361
Nov, 70	"	Kennington	"	0.55453
Nov, 71	*P. nigra*	Thetford	Root fresh weight/shoot fresh weight	0.93426
Nov, 70	"	Kennington	"	0.76237
Nov, 70	"	Wareham	"	0.87372
Nov, 71	*P. sylvestris*	Thetford	"	0.80438
Nov, 70	"	Kennington	"	0.92524
Nov, 70	"	Wareham	"	0.78246

b) Assessment of the form and development of mycorrhizal roots.

In order to understand the form, the development and the assessment of the mycorrhizal roots of young nursery grown trees of P. sylvestris and P. nigra, it was necessary to compare mycorrhizal roots with their mature counterparts.

Sampling was carried out at monthly intervals on selected Pines growing in Thetford Forest (to compare the seasonal variations in the form and development of mycorrhizal roots). To obtain a reference for the various seasonal changes of mycorrhizal form, characteristic mycorrhizal material was photographed and mycorrhizas confirmed by microscopic examination of cut sections. Comparison of the morphology of mycorrhizal roots obtained from pines growing in forest plots 458FY70 and 83FY70 with this reference collection of photographs helped decide on suitable root sampling times, the method of root sampling and any particular definition of mycorrhizal morphology.

Careful attention was paid to the interpretation of coralloid mycorrhizas; in particular an attempt was made to see whether this coralloid form originated from the development of dichotomous mycorrhizas, or from another source.

c) Fixation and sectioning of mycorrhizas.

It was sometimes difficult to distinguish between mycorrhizal and non-mycorrhizal dichotomous roots and so a proportion of mycorrhizal roots were fixed, sectioned and stained for examination by light microscopy. Dichotomous roots of P. sylvestris or P. nigra, either fresh or stored at 4°C, were carefully cleaned in running tap water and transferred to either distilled water, glutaraldehyde (2.5%; pH 7.3) or FAA (formalin: glacial acetic acid: alcohol (70%); 5: 5: 90 (v/v)).

Roots transferred to either FAA or glutaraldehyde were stored for the purposes of morphological examination and identification. Roots in distilled water were blotted dry and embedded in 3.5% Oxoid Ion Agar No. 2 at 50°C; the agar was then cooled quickly to 4°C. Embedded mycorrhizal roots were trimmed to 5mm cubes and frozen at -70°C to an ice face formed on the chuck of a Cambridge Rocking Microtome. After 5 minutes freezing the block was trimmed to expose the cutting face and placed in a Beck cryostat chamber at -30°C. Sections 6-8 μm thick were cut and mounted on clean glass slides.

Sections were stained for 15 minutes in an aqueous solution of Methyl Green: Pyronin (0.75%: 0.15%); rinsed in distilled water; progressively dehydrated in alcohol and mounted in Euparol. Some sections were stained but not dehydrated; these were mounted in distilled water.

Sections were also examined by phase contrast microscopy.

2. THE EFFECT OF SIMAZINE ON THE MYCORRHIZAL ROOTS OF P. SYLVESTRIS AND P. NIGRA.

To assess the effect of soil applied herbicides, mycorrhizal roots of seedling trees growing in herbicide treated soils can be compared with mycorrhizal roots of seedling trees growing in herbicide free soils.

In order to assess both the residual and direct herbicide effects on the mycorrhizal roots of P. sylvestris and P. nigra the herbicide must be continuously applied. Since this is not practicable, assessment of different application rates of herbicide usually consists of:

a) The assessment of the direct effect of the herbicide (this usually takes place soon after the period of application).

b) The assessment of the residual effect of the herbicide (this usually takes place before the period of herbicide reapplication).

The determination of soil site, seasonal and other environmental effects on Pine root systems cannot be properly assessed; to minimise this problem seedling pines were taken from statistically designed nursery blocks, with at least four replicated tree plots for each herbicide treatment. Using such treated tree plots it should be possible to separate the effect of the herbicide on the mycorrhizal roots from other site factors.

Since soil type is a direct reflection of environmental and physical conditions, different soil types may influence the nature of the herbicide effect on developing mycorrhizal roots.

a) Assessment of the effect of soil applied Simazine on the mycorrhizal roots of *P. sylvestris* and *P. nigra*.

The assessment of the effect of Simazine on *P. sylvestris* and *P. nigra* consisted of two continuous experiments; one in Forestry Commission nursery plots, the other in plant pots at Bedford College.

The trees used were from Forestry Commission growth experiments carried out in nursery plots at Kennington and Wareham. Sampling times were from October to February only; to assess the periods of spring and summer growth the trees were sampled in October or November. A single assessment for *P. nigra*, growing in the Wareham Forest nursery, showed that uninfected root tips and mycorrhizal roots were fewer in February than November. Seedlings of *P. sylvestris* and *P. nigra*, in 5" plant pots containing (a) chalky loam (pH 6.5) from Thetford Forest, (b) heavy clay loam (pH 6.2) from Wilmington Forest, (c) sandy loam (pH 5.8) from Wareham Forest, were grown at Bedford College. These trees were used for experiments to demonstrate the effects of different soil types on the morphology and development of the Pine root systems; and to see if this soil effect masked any effect of different applications of Simazine.

b) Design of the experiment to assess the residual effects of Simazine on *P. sylvestris* and *P. nigra* growing in nursery conditions at Wareham Forest and Kennington.

The forest nursery plots at Kennington and Wareham were ones which had been used previously for "The Weed Control In Nurseries Project" (No 320) of the Forestry Commission. The object of the above experiment by the Forestry Commission was to determine the effect of repeated applications of Simazine on the shoot growth of various coniferous species (see Figs. 1 and 2).

For this experiment Forestry Commission staff had planted plots in early March 1970 and 1971 with two year old seedlings of various coniferous species; including *P. sylvestris* and *P. nigra*. The conifers were planted in six foot rows (in plots each measuring four sq. yards), the rows being nine inches apart and the individual trees at two inch spacings (see Figs. 1 and 2). In late March 1970 and 1971, Simazine, i.e. "Weedex 50", was sprayed between the newly planted trees. Rates of application were 0, 1, 2, 4, and 8 lbs Simazine per acre. Application rates for each plot corresponded to the preceeding yearly applications for that plot.

Trees from this Forestry Commission experiment were taken for the present experiment to assess the residual effect of Simazine. Twenty of the centre plants of each row of *P. sylvestris* and *P. nigra* (together with some soil surrounding their roots) were carefully removed, placed in plastic bags, and taken back to Bedford College for assessment.

Table 3.

The following is a brief synopsis of the treatments and other routines used on the experimental plots 458FY70 and 83FY70 by the Forestry Commission.

<u>Prior to tree planting</u>:

1. 10 lbs per sq. yard of well rotted hop waste (75%) and bracken (25%) was applied to the surface of each 4 sq. yard plot.

2. Superphosphate (NPK- 0:20:20) was applied to the surface of the compost at the rate of 55 g per sq. yard, and 'Ciserite' (Mg) at the rate of 17 g per sq. yard.

3. The compost and added fertiliser were forked into the top 6 - 8 inches of the soil.

<u>On tree planting</u>:

4. In early March seven trenches 8 - 9 inches soil depth were dug across the experimental plot and the roots of conifers placed into each trench two inches apart.

5. The tree roots were sprayed with BHC (Strobol,at a concentration of 0.5 ml/l).

6. The trenches were refilled with their soil and the tree roots compacted.

<u>After planting</u>:

7. In late March the tree plots were sprayed with Simazine at the rate of either 0, 1, 2, 4, or 8 lbs per acre.

8. Dressings of "Nitro Chalk" were applied monthly from June to September at the rate of 22 g per sq. yard.

9. In early September height assessments of these trees, together with assessments of effective weed control, were undertaken by Forestry Commission staff.

10. In November <u>P.nigra</u> and <u>P.sylvestris</u> seedlings were taken

(Cont.)

Table 3. (Cont.)

to Bedford College for further assessment, and the remaining seedlings transplanted by the Forestry Commission.

On arrival at Bedford College the seedlings were carefully washed in running water, repacked in plastic bags and relabelled. The seedlings were stored at 4°C until assessed. This assessment took place over the following fortnight.

Assessment for the 1970 sample was as follows:

Fresh and dry weights of the root.

Number of mycorrhizal roots.

Number of non-mycorrhizal short roots.

Number of dichotomous non-mycorrhizal roots.

Assessment for the 1972 sample was as follows:

Fresh weights of the shoot and root.

Number of mycorrhizal roots.

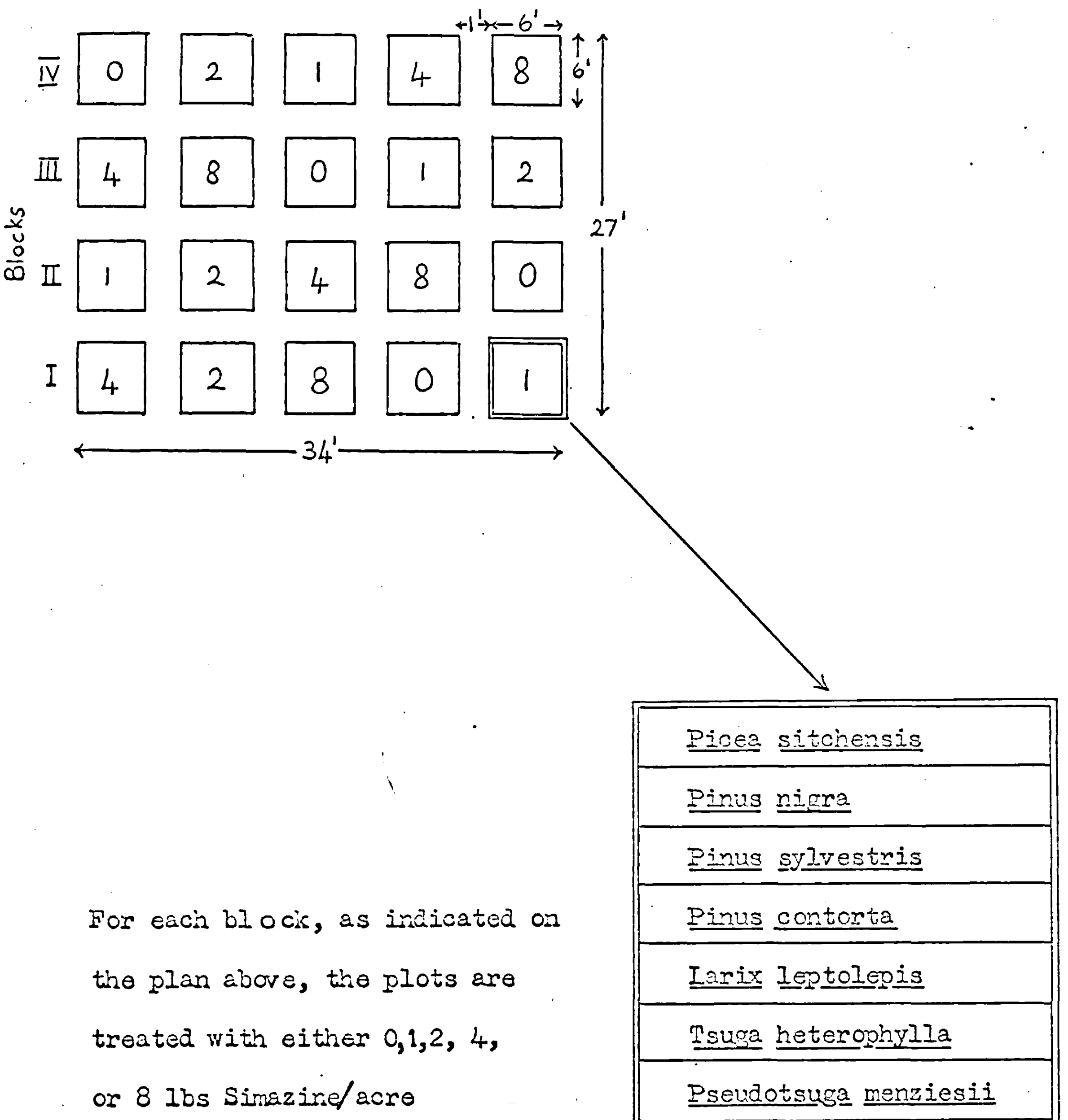
Number of non-mycorrhizal short roots.

Number of 'pioneer roots' (those with very few short root branches).

Number of 'mother' and 'subordinate mother' roots ('subordinate mother' roots are even smaller in diameter and more densely branched than 'mother' roots).

Figure 1. Plan of the Forestry Commission's Wareham nursery experiment

(designated as 83FY70)

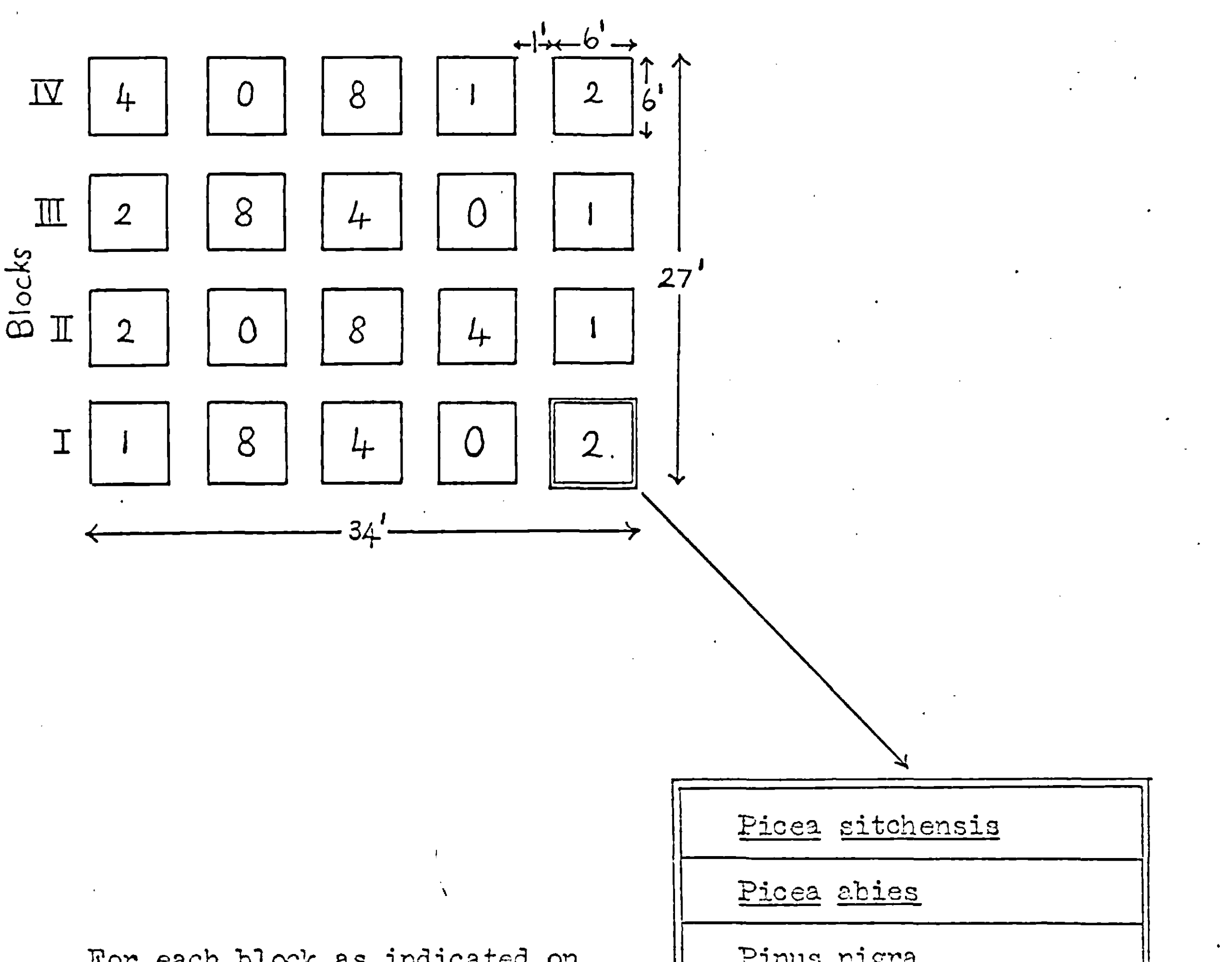


For each block, as indicated on the plan above, the plots are treated with either 0,1,2, 4, or 8 lbs Simazine/acre

Layout of coniferous species in one 6' x 6' plot.

Only *P. sylvestris* and *P. nigra* were taken for assessment.

Figure 2. Plan of the Forestry Commission's Kennington nursery experiment (designated 458FY70)



For each block, as indicated on the plan above, the plots are treated with either 0,1,2, 4, or 8 lbs Simazine / acre.

Layout of coniferous species in one 6' x 6' plot.

Only *P. sylvestris* and *P. nigra* were taken for assessment.

c) Design of an experiment to assess the effects of Simazine on P.sylvestris and P.nigra grown in different soil types in a controlled pot experiment.

(i) Nursery soils.

Samples of surface soil which had been routinely treated for ten years with application of 4 lbs Simazine per acre by the Forestry Commission, were separately collected from Forestry Commission plots 83FY70 (Wareham), and 458FY70 (Kennington) in November 1970, together with untreated soils. Each soil was dispensed in 5" plastic pots (500 mls/pot).

Ten two-year-old plants of P.sylvestris and P.nigra were potted in each of the Simazine treated soils. Twenty two-year-old plants of P.sylvestris and P.nigra were potted in soils that contained no Simazine. The pots were randomly distributed on a gravel bed with an eighteen inch spacing. In March 1971 and March 1972 the pots were each treated with the relevant quantity (16mg)of Simazine (equivalent to an application of 4lbs Simazine per acre).

(ii) Forest soils.

Quantities of surface soil from Brandon (Thetford) Forest were collected in November 1970. Forty plants of both P.sylvestris and P.nigra were potted individually in the soil with in 5" plastic pots each containing 500 mls soil. Simazine was applied at the rate of 16 mg. per pot (equivalent to 4 lbs Simazine per acre) to the surface of half the pots, and the remaining pots left untreated. The pots were randomly distributed on a gravel bed with

an eighteen inch spacing.

In March 1972 a further 16 mg. of Simazine was applied to each pot. (No organic or inorganic fertilisers or BHC were used in these experiments).

Plant pots (5") treated at the rate of 4 lbs/acre Simazine were weed-free but untreated plant pots were weeded occasionally. The purpose of this weeding was to minimise any differences between the levels of soil nutrients in the plant pots and to prevent the weeds chcking the pines.

The assessment of the nursery and forest soil pots was undertaken in September 1972 and was as follows:

Fresh weight of root and shoot.

The number of short roots/ gram 'mother' root.

The number of mycorrhizal roots.

The number of 'pioneer' roots.

The number of 'mother' and 'subordinate mother' roots.

Total shoot height.

Annual shoot growth.

The number of branches grown each year.

Length of shoot branches grown each year.

Overall growth of shoot branches.

3. THE ISOLATION OF ECTOTROPHIC MYCORRHIZAL FUNGI AND ROOT PATHOGENIC FUNGI.

Fungi known to form mycorrhizas or to parasitise the roots of either P.nigra or P.sylvestris were isolated in a variety of ways.

Pure cultures of these isolated fungi were used to study herbicide effects on their *in vitro* growth, to synthesise mycorrhizas, and to inoculate plant pots containing either P.sylvestris or P.nigra.

a) The isolation of ectotrophic mycorrhizal fungi
(i) from ectotrophic mycorrhizal roots.

Excised roots taken from pure stands of P.sylvestris or P.nigra were washed and placed in a 250 ml conical flask containing a trace of "Tween 20", and 50 ml distilled water. The mycorrhizal roots were agitated for 30 minutes, then transferred to 30 mls 100 vols hydrogen peroxide. After ten minutes immersion the hydrogen peroxide was removed and the roots washed with sterile water. Individual mycorrhizal roots were transferred to agar plates (basic ion agar).

Alternatively, the surface sterilised fungal cortex was dissected off the mycorrhizal roots and fragmented with sterile glass beads. The mycorrhizal material was washed with sterile water and the fragments incorporated into nearly solidified agar; alternatively the fragments were transferred to Petri dishes containing 40 mls agar. Incubation was at 25°C; specially prepared isolation plates incorporating Neomycin and a systemic fungicide (Benomyl) for the

selective isolation of basidiomycete mycelia (Taylor, 1971) were not usually necessary.

(ii) from germinating basidiospores.

Germination of dried collections of basidiospores was low or completely unsuccessful. Freshly collected spores germinated more successfully.

Portions of fresh sporocarp tissue kept overnight at 4°C were attached with sterile glycerine to the rubber washer inside a McCartney bottle top so that the spore or gill surface was exposed; the whole assembly was screwed onto a McCartney bottle containing sloped basic ion agar. After 48 hours at room temperature the bottle tops and attached sporocarp tissue were replaced by normal sterile bottle tops; the McCartney bottles were incubated at 25°C until spore germination occurred.

(iii) from sporocarps.

Young sporophores were collected from the sites listed in Table 4, and kept overnight at 4°C. Inner stipe or pileus tissue was aseptically removed and incubated in Petri dishes on basic ion agar.

In the case of Thelephora terrestris, sporocarps were surface sterilised with 100 vols hydrogen peroxide for ten minutes and left at 4°C. After a week at this temperature these sporocarps produced fresh tissue. This was aseptically removed and incubated at

25°C in Petri dishes on basic ion agar.

b) The isolation of root pathogenic fungi.

Sporocarps of *Fomes annosus* and *Armillarea mellea* were collected and kept overnight at 4°C. Pileus tissue was aseptically removed and incubated in Petri dishes on basic ion agar.

Wood infected with *Fomes annosus* was collected from *Pinus sylvestris* growing in Thetford Forest; the wood was surface sterilised with 100 vols hydrogen peroxide for 30 minutes and carefully broken apart with a sterile scalpel. Fragments of the wood were incubated at 25°C in Petri dishes containing basic ion agar.

Rhizomorphs of *Armillarea mellea* were collected from under the bark of *P. sylvestris*; these rhizomorphs were surface sterilised for 30 minutes with 100 vols hydrogen peroxide and aseptically sliced into long thick strips. These strips were incubated at 25°C in Petri dishes on basic ion agar.

Table 4. The fungal cultures and the means of their isolation.

Fungus	Source of isolate	Means of isolation
Amanita rubescens	Swinley (Surrey), under *P. sylvestris*	Sporocarp and basidiospores
Boletus bovinus	Windsor Park (Surrey), under *P. sylvestris*	Sporocarp
Boletus edulis	Epping Forest (Essex), under *P. sylvestris*	Sporocarp
Boletus elegans	Windsor Park, under *Larix* sp. but near *P. sylvestris*	Sporocarp and basidiospores
Boletus granulatus	Swinley, under *P. sylvestris*	Sporocarp and basidiospores
Boletus luteus	Thetford (Norfolk), under *P. sylvestris*	Sporocarp and basidiospores
Boletus variegatus	Swinley, under *P. sylvestris*	Sporocarp
Boletus testaceoscaber	Swinley, under *Betula* sp.	Sporocarp and basidiospores
Cenococcum graniforme	Culture Collection (Baarne)	-
Cenococcum graniforme?	Wareham (Dorset), under *P. sylvestris*	Mycorrhizal roots
Russula emetica	Swinley, under *P. sylvestris*	Sporocarp, basidiospores and Bedford College culture collection
Thelephora terrestris	Swinley, under *P. sylvestris*	Sporocarp and E.Hacskaylo (c/o U.S.D.A. Beltsville, U.S.A.)
Armillaria mellea	Thetford and Swinley, under *P. sylvestris*	Tree roots, rhizomorph, sporocarp or Forestry Commission culture collection (Alice Holt Lodge, Hants.)
Fomes annosus	Thetford, under *P. sylvestris*	Tree roots, or Forestry Commission culture collection
Peniophora graminis	Forestry Commission culture collection	-
Mycelium-radicis atrovirens	Culture Collection (Baarne)	-

4. THE IN VITRO STUDY OF THE GROWTH OF FUNGAL ISOLATES.

a) The media

Both basic ion agar and basic liquid media (Melin, 1954) were used for in vitro studies and for the isolation of mycorrhizal and pathogenic fungi. The basic liquid media consisted of:

Glucose	20g	$ZnSO_4 . 7H_2O$	5.0mg
Ammonium tartrate	0.5g	$FeSO_4 . 7H_2O$	5.0mg
KH_2PO_4	1.0g	$MgSO_4 . 7H_2O$	0.5mg
Thiamin hydrochloride	0.05 mg		

- these salts, of Analar grade, were dissolved in distilled water to one litre. 12.0g of Oxoid Ion Agar No.2 added to the salts (before making up to one litre) gave basic ion agar. Basic ion agar and basic liquid media were steamed to dissolve the constituents and autoclaved at 15 lbs psi steam pressure for 15 minutes.

b) The selection of cultures for experiment

Cultures isolated from sporophores or obtained from culture collections were positively identified and were preferred for this reason. Cultures of Armillarea mellea obtained from rhizomorphs and cultures of Fomes annosus obtained from diseased timber were also positively identified.

Examination of the fungi isolated from mycorrhizal roots was difficult; mycelial features such as clamp connections for basidiomycetes were not readily observed. Due to lack of an adequate reference culture collection, identification using the

hyphal fusion technique was impracticable.

Fungi isolated from the mycorrhizal roots of *P. sylvestris* and *P. nigra* were used to inoculate aseptic *P. sylvestris* or *P. nigra* seedlings growing in 2l Erlenmeyer flasks containing Vermiculite and nutrient solution (see Section 7). Although abundant mycelial development occurred in the Vermiculite, mycorrhizas were not formed. In one case the fungus isolated from the mycorrhizal roots of *P. sylvestris* closely resembled known isolates of *Cennococcum graniforme*. However, this isolate rapidly parasitised aseptic seedlings of *P. sylvestris*.

c) The *in vitro* growth rate of fungal isolates.

Colony growth of fungal isolates measurably declined when the fungal isolates were continuously subcultured. However, the same fungi produced consistent growth rates if subcultured, when required, from isolates maintained on slopes under a layer of sterile paraffin oil. Isolates were initially grown on basic ion agar plates at 25°C (Table 4).

Once the mycelia successfully colonised the agar surface the fungal isolates were subcultured. The subcultures were stored on slopes at 4°C at room temperature or under a layer of sterile paraffin oil. The mycelial morphology of all isolates was examined on basic ion agar. Isolates of the same fungus often showed varying characteristics and it was important to select isolates that on subculture did not sector (that is change in either morphology or growth rate). To ensure that subcultured mycelia was actively

growing, it was equally important to select mycelial tissue (for subculture) from the periphery of the growing colony, and to check that subcultures were not derived from sectors.

Those isolates which grew easily, giving reproducible growth rates, were chosen for further experiment. Many isolates, especially those of Boletus badius, and one particular isolate of Cenococcum graniforme, would not grow across the surface of basic ion agar; instead these isolates produced compact colonies around their central inoculum and made assessment of colony growth on agar impossible.

For these reasons, isolates of Russula emetica, Cenococcum graniforme and M. radicis atrovirens were chosen from cultures available, and other chosen isolates were derived from mycorrhizal roots.

5. THE SOLUTION OF HERBICIDES.

Herbicides (Table 5) were made up at 25°C to the following concentrations in aqueous solution.

Simazine	5 ppm
Atrazine	30 ppm
245T	250 ppm
Chlorthiamid (Prefix)	2700 ppm
Diclobenil	25 ppm
2,6 Dichlorobenzoic acid	550 ppm

In practice, these solubilities were only approached with difficulty after long periods of agitation on a reciprocal shaker at 25°C.

Since there is a linear relationship between absorbance in the ultra-violet and the concentration of the chemical in solution (The Lambert Beer law), the final herbicide concentrations can be checked by recording the absorption maxima in the U.V. and comparing these maxima with previously prepared calibration curves (Fig 3).

The wavelengths for maximal absorbance within the U.V. range are: 245T - 202 µm; Simazine and Atrazine - 220 µm; Prefix and 2,6 Dichlorobenzoic acid and Dichlobenil - 208 µm.

Sterility of these herbicide solutions is important. Diclobenil and 2,6 Dichlorobenzoic acid can be formed by the microbial degradation of Chlorothiamid.

Herbicide solutions could not be stored at 4°C due to the lowering of their solubilities at this temperature (and their

consequent precipitation); hence herbicides were filter sterilised with an Oxoid Membrane Filter (No 2) and stored at 25°C in sterile medical flats.

FIG. 3.

CALIBRATION CURVES FOR THE ABSORBANCE OF DIFFERENT HERBICIDES AT THE WAVELENGTHS INDICATED.

245T, 202nm.
Diclobenzoate, 208nm.
Chlorthiamid, 208nm.
Dichlobenil, 208nm.
Atrazine, 220nm.
Simazine, 220nm.

Absorbance.

0·1
0·3
0·5
0·7
0·9

1
2
3
4
5
6
7

Concentration of herbicide(ppm).

Table 5. The names, formulae, solubilities and sources of herbicides used.

Name	Formula	Stated Solubility at 25°C	Source
Simazine	2 chloro - 4,6 bis(ethylamino) 1,3,5 triazine Cl; N N; C_2H_5NH NHC_2H_5; N	5 ppm	Geigy (UK Ltd). Simonsway Manchester
Weedex '50'	50% Simazine	contains wetting agent	Fisons Ltd Harston Cambridge
Atrazine	2 chloro - 4 ethylamino 6 isopro-pylamino 1,3,5 triazine Cl; N N; C_2H_5NH $NHCH(CH_3)_2$; N	33 ppm	Geigy (UK Ltd) Simonsway Manchester
245T	2,4,5 Trichloro phenoxy acetic acid CH_2COOH; O; Cl; Cl; Cl	251 ppm	Plant Protection Ltd Jeallots Hill Bracknell, Herts
Prefix (Chlorothiamid)	2,6, Dichlorothiobenzamide S=C-NH_2; Cl Cl	2,700 ppm	Shell Research Ltd Sittingbourne Kent

Table 5. (Cont.)

Dichlobenil	2,6, Dichloro-benzonitrile C≡N Cl Cl	25 ppm	Shell Research Ltd Sittingbourne Kent
Dichlo- benzoate	2,6, Dichloro-benzoic acid C=O OH Cl Cl	850 ppm	Shell Research Ltd Sittingbourne Kent

6. ASSESSMENT OF HERBICIDE EFFECT ON THE *IN VITRO* GROWTH OF FUNGAL ISOLATES.

a) Assessment of herbicide effect using agar plates

Basic ion agar was pipetted into Petri dishes containing 2 ml aliquots of aseptic herbicide to give the required herbicide concentration and a final agar volume of 20 ml. The Petri dishes were gently agitated to ensure thorough mixing of the herbicide and agar.

Final concentrations of the herbicides in the Petri dishes were:

Herbicide concentration

(ppm.)

Simazine	Atrazine	245T	Diclobenil	Chlorth-iamid	Dichloro benzoic acid	
0.5	3.0	25.0	2.5	85.0	42.5	
0.25	1.5	12.5	1.2	42.5	21.25	
0.12	0.75	6.25	0.6	21.2	10.62	
0.06	0.37	3.12	0.3	10.6	5.31	
0.03	0.18	1.66	0.15	5.3	2.65	
0.015	0.09	0.83	0.07	2.6	1.32	
0.007	0.04	0.41	0.035	1.3	0.66	
0.003	0.02	0.20	0.017	0.65	0.33	
0.0	0.0	0.0	0.0	0.0	0.0	Control

(Controls consisted of 2 ml aliquots of sterile water instead of biocide).

When set, the agar plates were inoculated with 5mm plugs of mycelium taken from the periphery of an actively growing mycelium incubated at 25°C on basic ion agar. Hence the rate of growth for the

fungal isolate growing on different biocides could be compared.

Each treatment was replicated five times; the replicate plates were carefully distributed through the incubator to allow for temperature variations. The pH of the agar was checked before and after the experiments and was found to be unchanged. The experiments continued until the colony growth plotted with respect to time presented either little or no further growth, or until the colony radius approached that of the Petri dish.

Occasionally differences in the growth rate of an isolate occurred; this was due to differential growth (sectoring) of the mycelium and was compensated for either by repeating the experiment, or, when the Petri dishes affected were few in number, by excluding affected cultures from the results.

The diameter of each colony was measured along a centrally drawn line on the underside of each Petri dish. Contaminated plates were discarded; badly contaminated treatments were repeated.

Growth rates were expressed as the radial increase of colony size (mm) / time (hours). The initial inoculation plug was 5mm diameter, consequently colony growth was expressed as:

$$\frac{(\text{Total diameter of colony (mm)} - 5\text{mm})}{2} \text{ mm.}$$

Concurrent experiments using six herbicides together with eight dilutions of each herbicide and full replication for each fungal isolate usually produced replicable results. These results were used to define the herbicide effects on the colony growth of each fungal isolate.

b) Assessment of herbicide effect using liquid cultures

The fungal isolates used for the liquid culture experiments were the same ones used for the assessment of herbicide effects on basic ion agar. In still liquid culture, colony growth was often affected by the cultures sinking rather than the inhibitory effect of the herbicide contained in the basic liquid media. In shake liquid cultures the same cultures of the fungal isolates were often difficult to assess due to irregular fragmentation of the mycelial pellets.

Basic liquid media (see section 4), 10/9 of the required strength, was sterilised (15 lbs psi steam pressure for 15 minutes) in 100 ml conical flasks. When cool, 2 ml aliquots of aseptic herbicide were added to each flask to give the required herbicide concentrations and a final volume of 20 ml per flask. Final concentrations of the herbicides in the conical flasks corresponded to the agar plate experiments (section 6a). Eight aseptic herbicide dilutions were used; these were replicated five times and the controls ten times. Each flask was inoculated with one quartered 5 mm plug of mycelium stripped of surplus agar. The flasks were placed in an incubator or a reciprocal shaker at 25°C for three weeks.

No attempt was made to duplicate completely the agar plate experiments. Instead, certain fungus/herbicide combinations were examined on the basis of results obtained with the agar plate technique. After three weeks incubation the mycelium was removed

from the basic liquid medium, washed, suction filtered on tared filter paper, and oven dried at 90°C to constant dry weight.

Results were expressed as mean mycelial dry weight (after three weeks incubation) for each herbicide concentration.

7. THE SYNTHESIS OF MYCORRHIZAS.

Techniques used by Melin (1921), Trappe (1967b) and Fortin (1966) were followed or adapted for the experimental synthesis of mycorrhizas.

a) Melin and Hacskaylo's technique.

Seeds of *Pinus sylvestris* and *Pinus nigra* obtained from Forestry Commission Research, Alice Holt Lodge, Bentley, Hants, were surface sterilised for thirty minutes in 100 vols hydrogen peroxide containing a trace of 'Tween 20'. The seeds were placed on basic ion agar and incubated at 25°C; about 90% germinated within 10 days.

Ten-day-old aseptic seedlings were each transferred to 2 litre flasks containing 30 g of medium grade Vermiculite and 250 ml of a nutrient solution which contained:

Glucose	2.5g	$CaCl_2$	0.05g
$(NH_4)_2HPO_4$	0.25g	NaCl	0.025g
KH_2PO_4	0.5g	EDTA (Fe salt)	6.0mg
$MgSO_4.7H_2O$	0.15g	Thiamin HCl (added with the fungus)	0.015mg
		Distilled water to one litre	

In agreement with the findings of Hacskaylo (1953) and Ferry (unpublished), the pH of the media was raised considerably after autoclaving (to about pH 7.5) and consequently HCl was added to each flask to give a pH of 4.5. In some experiments sieved and non-sieved mycelial suspensions (at 1.0 ml per flask) were used to inoculate the flasks, in other experiments each flask was inoculated with mycelia

stripped from four 5 mm agar plugs.

The mycelial suspension was prepared by homogenising mycelia with glass beads in sterile distilled water. To obtain a sieved suspension the material was strained through fine-meshed muslin gauze.

Flasks were inoculated with the fungus when the pine seedlings were either 2 or 13 weeks old. When the seedlings were about 12 or 20 weeks old respectively they were removed from the flasks, washed to remove the Vermiculite and examined for mycorrhizal roots.

Roots selected for further examination were carefully washed in tap water and transferred to FAA fixative for further examination (see section 1c).

b) <u>Trappe's technique</u>

Trappe's technique consists of an assembly for growing tree seedlings, so that the roots are in aseptic conditions, while the aerial parts are in the open. Wide mouthed specimen jars, about 5 cms diameter and 7 cms height, were brim-filled with either acid washed sand or Vermiculite; various nutrient solutions (see Table 6) were added at the same rate as in (a) above. A glass tube 2 cms in diameter and 2 cms deep was inserted into the Vermiculite or sand so that the top of the tube was level with the jar top. Material in the tube was removed, the jar top covered with aluminium foil and fastened with light-weight galvanised wire. The whole assembly

was autoclaved for 30 minutes at 15lbpsi steam pressure.

One of the purposes of the glass tube was to concentrate the fungal inoculum around the seedling roots. It also served to reduce the compaction of the Vermiculite which results from autoclaving.

After swabbing with alcohol the aluminium foil was punctured with a sterile needle over the cavity excavated inside the glass tube insert. Through this hole a 2 - 3 cms long sterile radicle of a P. sylvestris or P. nigra seedling was carefully inserted. The hole was sealed using rubber solution (the benzene solvent maintained sterility until the rubber hardened).

The seedlings were allowed to grow in subdued light and the glass jars checked for visible contaminations. Once the seedlings were established another puncture, near the developing shoot, was made with a sterile hypodermic needle, and an inoculum of 0.5 mls sieved mycelial suspension introduced. This puncture was sealed with rubber solution and, to exclude light, the jar was wrapped with aluminium foil.

Seedlings were allowed to grow for up to eight months, or, in certain cases, longer. At the end of this period any mycorrhizal roots formed were transferred to FAA for later examination.

Table 6. Trappe's (1967) nutrient medium for the synthesis of mycorrhizas and the nutrient medium as modified for some of the present experiments.

Trappe's media:

5.0g	glucose	0.15g	$MgSO_4.7H_2O$
0.25g	KH_2PO_4	1.2ml	1% Ferrous ammonium citrate
0.125g	$(NH_4)_2HPO_4$	50 mg	Thiamin
0.05g	$CaCl_2$		Distilled water to 1 litre

Where the modified technique was employed, the nutrients were as follows:

Glucose (g)	Thiamin (mg)	KH_2PO_4 (g)	$(NH_4)_2HPO_4$ (g)	Final pH of media Sand	Vermiculite
0.1	50.00	0.250	0.125	5.0	6.7
		0.125	0.250	5.2	7.0
	0.05	0.250	0.125	5.2	6.8
		0.125	0.250	5.2	7.1
1.0	50.00	0.250	0.125	5.0	6.6
		0.125	0.250	5.3	7.1
	0.05	0.250	0.125	5.2	6.7
		0.125	0.250	5.2	7.0

Eight variations in nutrient concentration were made; these were as above; other salts used by Trappe were not altered.

20 aseptic P. sylvestris seedlings were grown in each variant of the media.

c) Fortin's technique.

Aseptically germinated seedlings of *P. sylvestris* or *P. nigra*, whose hypocotyls were not swollen and whose roots were between 30 and 40 mm long, were selected for this technique of mycorrhizal synthesis (Fortin, 1966). The root meristem was dissected off and the shoot removed to leave an attached hypocotyl of approximately 5 mm. The hypocotyls were inserted into 12 x 35 mm glass shell vials containing 0.5 ml sucrose/agar media (see Table 7). The whole assembly (i.e. glass shell vial and inserted root) was placed in a Petri dish containing 10 mls Vermiculite and 2.0 mls mineral media (see Table 7). Three drops of sieved mycelial macerate of the fungus were introduced over the pine root surface. After inoculation the Petri dishes were sealed with 'Sellotape' and incubated at 20°C for 60 - 70 days. The Petri dishes were opened and the degree of mycorrhizal infection assessed, together with the following:

1. pH of the Vermiculite.
2. Total length of the main root.
3. The number of lateral roots and their lengths.
4. The number of second order lateral roots.

Many of the root systems were photographed. In addition, transverse sections were cut to confirm the ectotrophic mycorrhizal association between host and fungus.

Table 7. The nutrients used for Fortin's technique for mycorrhizal synthesis.

1. The sucrose agar media (for the glass shell vial into which the hypocotyl is inserted).

Sucrose - 175.0 g
Thiamin - 1.0 mg
Choline chloride - 2.5 mg
Oxoid Ion Agar No. 2 - 10.0 g
Distilled water to 1 litre

The media was steamed and 0.5 ml carefully added to each glass shell vial. The vials were autoclaved at 15 lbs psi steam pressure for 15 minutes.

2. The mineral media (this is added to the vermiculite base).

$Ca(NO_3)_2.4H_2O$ - 72 mg
$MgSO_4.7H_2O$ - 20.5 mg
Distilled water to 1 litre
KH_2PO_4 - 10.0 mg
1% Ferric citrate solution - 1.0 ml
Trace element solution - 1.0 ml

3. The trace element solution (added to the mineral media).

KCl 3.728 g
H_3BO_3, 1.546 g
$MnSO_4.4H_2O$, 0.845 g
$ZnSO_4.7H_2O$, 0.575 g
$CuSO_4.5H_2O$, 0.125 g
$(NH_4)_6Mo_7O_{24}.4H_2O$, 0.018 g
Distilled water to 1 litre

8. HERBICIDES AND THE RESPIRATION RATES OF MYCORRHIZAL ROOTS.

Intact roots of pine were taken from the litter layer (pH 5.5) of a mature stand of Pinus sylvestris growing in Thetford Chase, Norfolk. The roots were stored between alternate layers of decaying needle litter in polythene bags at 4°C. Mycorrhizas were dissected off the stored pine roots, washed in running water and placed in 0.01 M phthalate buffer at pH 5.5. The roots, in buffer, were aerated with an air bubbler until required. Selected material consisted of coralloid mycorrhizas developing from successive second order lateral roots on a 'mother root' (see Figure 4). (Wilcox 1964, 1968 a & b; Sutton, 1969).

These mycorrhizal roots were blotted, weighed into 0.1 g samples and tied in dental gauze to form small bags, approximately 10 x 15 mm. These bags were placed in aerated phthalate buffer (pH 5.5).

A Clarke Oxygen Electrode (Yellow Springs, Ohio, USA) was used to record the respiration rate of the mycorrhizal roots, and to record the rate after treatment with various concentrations of herbicide.

Electrode sample chambers contained 10.0 ml 0.01 M phthalate buffer at pH 5.5; the buffer in the sample chambers was equilibrated in air at 20°C so that the solutions were fully oxygenated.

The degree of oxygen saturation, the temperature control, and the degree of drift of the pen recorder (due to either a faulty probe or to some malfunction e.g. air bubbles in the sample chamber) were all checked. When the recorder showed less than 1.0% drift per 10 minutes, the muslin bag containing mycorrhizal roots was introduced into the sample chamber. This muslin bag protected the mycorrhizal roots from maceration by the magnetic stirrer. In order to minimise the number of air bubbles in the sample chamber the muslin containing the mycorrhizal roots in the aerated phthalate buffer was placed in a vacuum desiccator, evacuated and left for a few minutes. If this mycorrhizal sample was quickly introduced into the sample chamber containing aerated buffer, and the probe inserted carefully, bubbles in the sample chamber were not usually a problem.

Herbicide concentrations (ppm.) used for the measurement of the respiration rates of mycorrhizal roots and mycorrhizal fungi.

245T	Dichlo-benzoate	Prefix	Dichlo-benil	Simazine	Atrazine
2.5	-	8.5	-	1.0	5.0
5.0	-	85.0	1.0	2.0	10.0
7.5	42.5	112.0	5.0	3.0	20.0
10.0	85.0	170.0	10.0	4.0	30.0
15.0	142.0	285.0	15.0	5.0	40.0
27.5	212.0	425.0	30.0	6.0	50.0
-	-	-	-	-	60.0

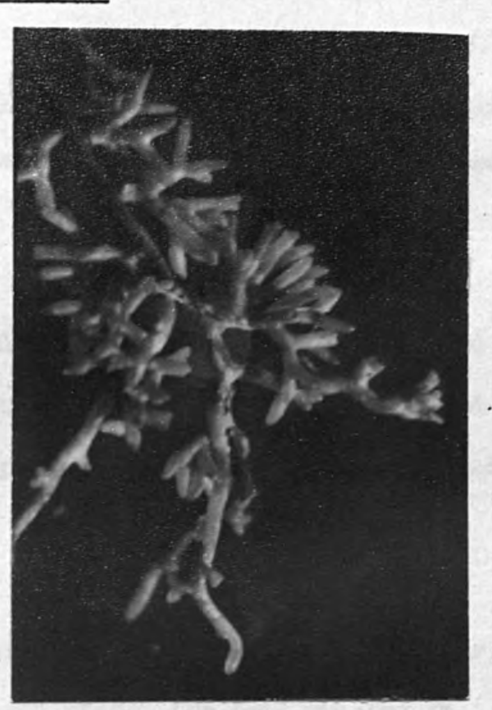
After the control respiration rate had been recorded the samples of mycorrhizal roots were placed in aerated herbicide/buffer solutions of increasing strength for 20 minutes, returned to herbicide-free buffer and allowed to recover for a further five minutes. The respiration rates were then recorded in the sample chamber contain-

-ing equilibrated fresh buffer.

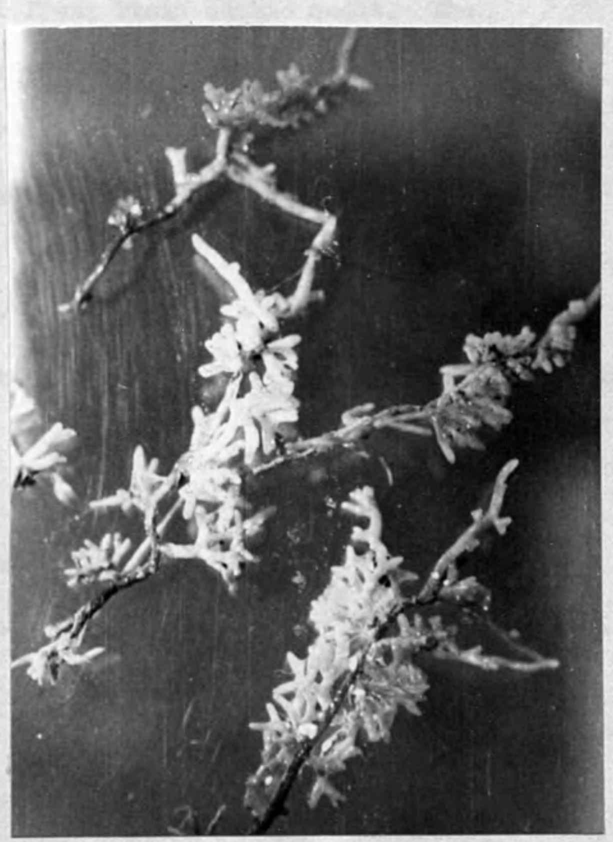
Results were expressed as μl O_2 consumed per 100 mg mycorrhizal root sample per minute.

Fig. 4.

Mycorrhizal samples used for respiration measurements with the Oxygen Electrode



Sample of mycorrhizal root



100 mg mycorrhizal root sample: for oxygen electrode or phosphate uptake techniques

9. HERBICIDES AND THE RESPIRATION RATES OF MYCORRHIZAL FUNGI.

The respiration rates of isolates of Cenococcum graniforme, Boletus variegatus, Thelophora terrestris, Boletus badius and Boletus elegans used for herbicide assessments were measured with an Oxygen Electrode.

Erlenmeyer flasks (250 ml) containing 50 ml basic liquid media were inoculated with two quartered plugs of mycelia stripped of surplus agar. The flasks were placed in a reciprocal shaker and incubated at 25°C for ten days.

After incubation the flasks were removed from the shaker, the pellets of mycelium taken out and placed for five minutes in 50 ml beakers containing 20 ml fresh basic liquid media. The respiration rates of individual pellets (blotted weight approximately 80 mg) were recorded (see previous section) in 7.0 ml fully aerated fresh basic liquid media at 25°C. The pellets were then placed in basic liquid media containing a known concentration of herbicide, aerated for ten minutes, removed, washed in fresh media for a further minute and the respiration rate re-measured. The pellets of mycorrhizal fungal tissue were unaffected by the magnetic stirrers.

Results were expressed as μl O_2 consumed per 100 mg fresh weight fungus. The range of herbicides examined was as in the previous section.

10. HERBICIDES AND THE UPTAKE OF PHOSPHATE BY MYCORRHIZAS.

The uptake of PO_4^{3-} by mycorrhizal roots was estimated by assaying the concentration of PO_4^{3-} remaining in the medium. The phosphate present in the buffer was determined by the method of Fiske and Subarrow (1925). The material selected consisted of highly developed coralloid mycorrhizas. The samples contained very little cut surface, little damaged tissue, and a large surface area of mycorrhizas (Fig.4).

Ten 100 mg samples of mycorrhizal material were each placed into test tubes containing 10 mls 0.01 M phthalate buffer (pH 5.5), 0.5 mg KH_2PO_4 and herbicide at various concentrations. The test tubes were placed in stabilised 50 ml Erlenmeyer flasks; bubblers (fed from a manifold supplying air at a constant rate) aerated both the buffer and the mycorrhizas. A low speed air flow maintained the temperature at 20°C and minimised evaporation of the buffer (evaporation would increase the concentrations of phosphate and herbicide).

0.2 ml aliquots of the buffer were taken and placed in clean dry test tubes; 1.0 ml distilled water was added to each aliquot. 1.0 ml 2.5% ammonium molybdate in 5N H_2SO_4 was added to each tube, the contents thoroughly mixed and 0.2 ml reducing agent, consisting of 0.25% 1, 2, 4 amino-naphthol-sulphonic acid in 0.5% sodium sulphite and 10% sodium metabisulphite solution, added. The sample was thoroughly mixed.

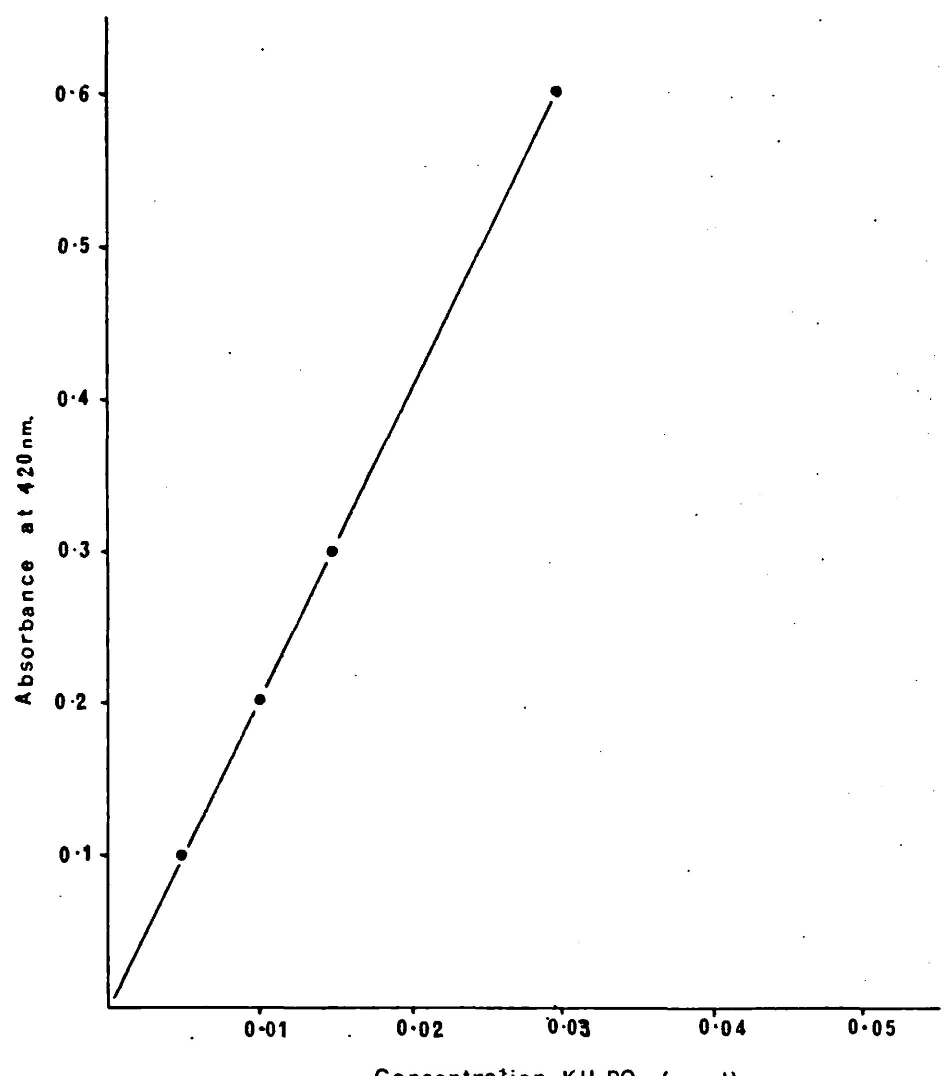
Normally the blue phospho-ammonium molybdate complex was fully developed ten minutes after the final mixing. The concentration of

phosphate was determined colourimetrically using a Beckman D. B. Grating Spectrophotometer at 420 nm. The readings were converted to mg PO_4^{3-}/ml using standard curves constructed from the extinction of known phosphate concentrations (Fig.5).

After the addition of H_2SO_4 and ammonium molybdate, each tube containing any Simazine or Atrazine developed a normal blue colour, but turbidity (caused by interaction of the added Simazine or Atrazine) interfered with assays of phosphate in solution. Some compounds are known to interfere with the colourimetric determination of inorganic phosphate(El-Dorry et al, 1971). Since Simazine and Atrazine are now known to interfere, they are now included in this category. Hence any effects of Simazine or Atrazine on the uptake of phosphate by mycorrhizas could not be observed colourimetrically.

FIG. 5.

CALIBRATION CURVE FOR THE ABSORBANCE OF PHOSPHATE SOLUTIONS AT 420 nm.



RESULTS

1. THE FORM AND DEVELOPMENT OF PINE ROOT SYSTEMS.

The root systems of the Pines examined were clearly heterorhyzic, in agreement with Wilcox's (1964) classification scheme; additionally it was possible to subdivide the woody, long lateral pine roots examined into Wilcox's classes of 'pioneer', 'mother' and 'subordinate mother' roots (see Tables 8, 10, 11 and 12).

Non-woody short roots were found to be less frequent on the root systems of P. nigra than P. sylvestris. Both species of pine growing in forest plots 458FY70 (Kennington) and 83FY70 (Wareham) developed few mycorrhizal short roots; in some cases the short roots were dichotomous but not mycorrhizal. However, short roots of P. sylvestris and P. nigra, growing in less fertile Thetford soils, or in more extreme environmental conditions (plant pots), were abundantly mycorrhizal compared with those of pines growing in Kennington and Wareham forest plots.

The results of both pot and field experiments show that P. nigra has fewer mycorrhizal tips and fewer non-mycorrhizal dichotomous root tips than P. sylvestris (Table 8). In addition P. nigra has more 'pioneer' roots, fewer 'mother' roots and fewer 'subordinate mother' roots than P. sylvestris. The total number of root tips per tree is less for P. nigra than P. sylvestris, as is the shoot and root fresh weight.

Table 8. The overall differences between assessed samples of two year old P. sylvestris and P. nigra seedlings.

(Results are expressed as weight (grams) or numbers per plant).

P. sylvestris	Field		5" plant pots	
	range	mean	range	mean
Shoot fresh weight	-	-	9 - 25	21.9
Root fresh weight	3 - 6	4.8	14 - 19	15.3
No. of lateral roots *	5 - 6	6.2	9 - 15	10.5
No. of 'pioneer' roots	0 - 2	1.3	1 - 2	2.3
No of root tips per gram root	69 - 162	102.8	72 - 173	101.6
Total no. of root tips	340 - 880	629.5	-	-
No. of mycorrhizas	8 - 173	32.9	55 - 241	169.3
No. of dichotomies†	2 - 28	19.2	0 - 4	2.3

P. nigra	Field		5" plant pots	
	range	mean	range	mean
Shoot fresh weight	-	-	10 - 16	14.25
Root fresh weight	2 - 4	3.7	7 - 10	10.2
No. of lateral roots *	3 - 5	3.8	4 - 10	7.4
No. of 'pioneer' roots	3 - 6	5.7	1 - 2	1.6
No. of root tips per gram root	45 - 140	80.2	23 - 74	50.4
Total no. of root tips	150 - 294	247.3	-	-
No. of mycorrhizas	5 - 21	13.7	10 - 26	21.7
No. of dichotomies†	2 - 36	16.2	0 - 1	0.06

* lateral roots are 'mother' and subordinate 'mother' roots.

† non mycorrhizal.

A study of the root systems of young self-sown pine seedlings developing in King's forest (Thetford Forest) and Thetford Chase showed that mycorrhizal infection took place within the first few weeks of growth. However young nursery seedlings, germinated in the same localities, were not usually mycorrhizal until the following year. Developing mycorrhizal systems on self sown pine seedlings were either unbranched, dichotomous, or coralloid in form.

In agreement with the findings of MacDougal and Dufrenoy (1944a); Wilcox (1964) and many other workers, the mycorrhizas were found to develop from non-woody short roots (often referred to as second order lateral roots) (Figure 14). An Hartig net surrounding the first order lateral roots necessarily infected any emerging short roots. Mycorrhizas formed in this way were always dichotomous.

The cycles of seasonal growth and development of mycorrhizas observed were in agreement with Aldrich Blake (1930) and Ladefoged (1939). Mycorrhizas grow in two seasonal cycles; that is late spring and late autumn. The following year these mycorrhizas may become moribund (Figure 17); new mycorrhizal development is confined to newly developed short roots. Hence it was important to discriminate between active and moribund mycorrhizas.

Evidence was accumulated to suggest that many mycorrhizal roots, especially coralloid ones, were not derived from infection of short roots by the Hartig net but from fungal propagules in the

soil. For example, mycorrhizal infection of 'pioneer' roots is infrequent (Figure 13) and results suggest that partly decayed root branches and pine cones either contain fungal propagules which are responsible for the mycorrhizal infection of these 'pioneer' roots or constitute a favourable environment for mycorrhizal formation (Figures 6, 7 and 8).

Although at many sites (Thetford, Wareham, Wilmington and Kennington) the mycorrhizal morphologies were identical, varying degrees of mycorrhizal infection were noted. Generally in one year four types of mycorrhiza were identified:

1) Non-dichotomous, racemose mycorrhizas found on roots that had penetrated pine cones; the pattern of the mycorrhizal roots complying with the shape and size of the cone segments (Figures 6 and 16).

2) Complex coralloid mycorrhizas found on 'pioneer' and 'mother' roots which had penetrated decaying root branches, pine cones or other organic material; these coralloid mycorrhizas often extended beyond the organic material (Figures 7 and 8).

3) Dichotomous mycorrhizas developed from the regular acropetal system of short roots. Such mycorrhizas may develop into a series of dichotomies and form small coralloid mycorrhizas (Figures 9, 10, 11, 14 and 15).

4) Figure 12 shows a very infrequent form of "mycorrhiza" which takes the form of a small white spherical structure. Such a structure seemed to develop from dichotomous mycorrhizas; it was found infrequently at all the sites examined.

The morphology of mycorrhizal roots developing on P. sylvestris.

Figure 6.
(Non-dichotomous, racemose)?
mycorrhizas found on roots which had penetrated pine cones. x2.

Figure 7.

Complex coralloid mycorrhizas found on 'pioneer' roots penetrating decaying root branches. x5.

Figure 8.

Complex coralloid mycorrhizas found on 'mother' roots penetrating organic material. x2.

Figure 9
(x2).

Figure 10
(x5).

Figures 9 and 10.

Dichotomous mycorrhizas developed from the regular acropetal system of short roots.

Figure 11.

Dichotomous mycorrhizal roots (Figures 9 and 10) may develop into small coralloid mycorrhizas. X 2.

Figure 12.

Small white spherical structures occasionally found on dichotomous mycorrhizas. X 5.

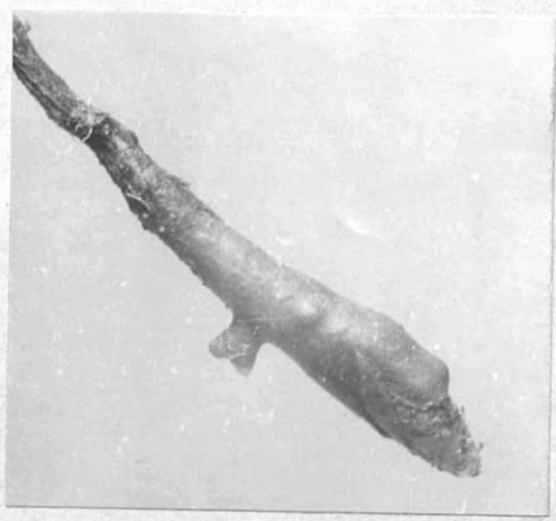


Figure 13.

Dichotomous mycorrhizas found on a 'pioneer' root tip. x 5.

Figure 14.

Dichotomous mycorrhizas forming second dichotomies on a 'mother' root. X 2.

Figure 15.

Simply branched mycorrhizas developing on a 'mother' root. X 5.

Figure 16.

Non dichotomous racemose mycorrhizas (developing in partly decomposed humus). X2.

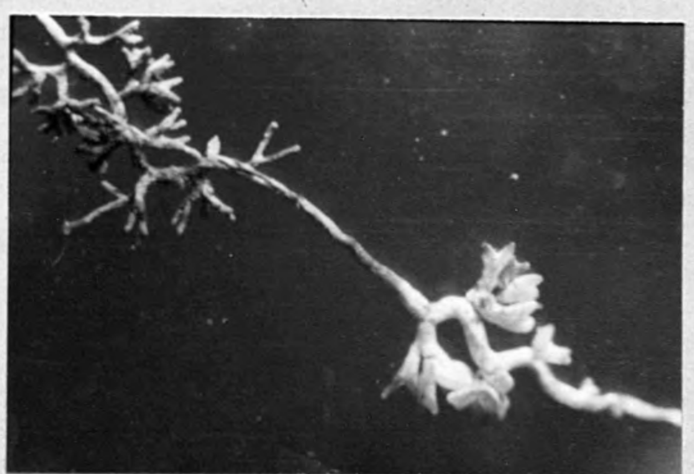
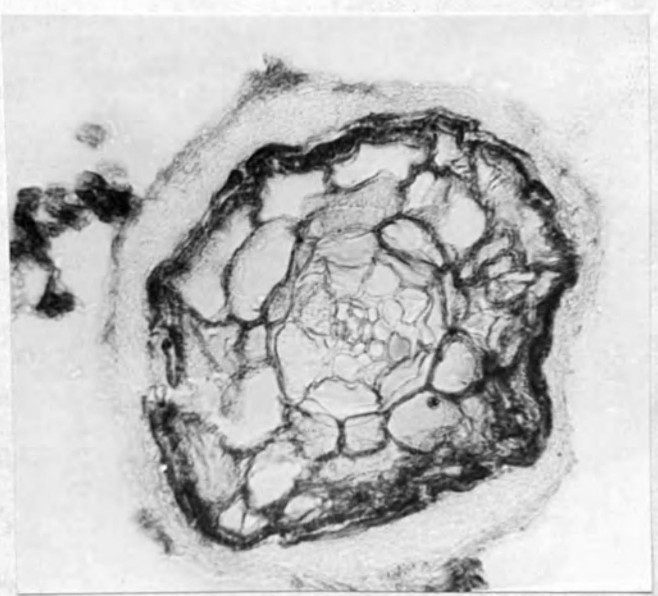


Figure 17.

Seasonal development of mycorrhizas on a 'mother' root. X2.

Figure 18. Transverse section of dichotomous mycorrhiza developed on a short root of P. sylvestris. (x250).



Transverse sections of mycorrhizas found on P. sylvestris roots confirmed their ectotrophic nature. All sections showed a well developed Hartig net surrounded by a fungal sheath.

2. THE POSSIBLE EFFECT OF SIMAZINE ON THE MYCORRHIZAL ROOTS ON P. SYLVESTRIS AND P. NIGRA

Simazine, although slightly affecting the mycorrhizas and uninfected short roots of P. sylvestris and P. nigra growing in forest plots 83FY70 (Wareham) and 458FY70 (Kennington), did not measurably influence any other aspects of the root systems of pine seedlings. However, a factor which confused the effect of Simazine appears in part of the Kennington site (Figure 2). This factor was revealed on assessment of the dry weight / fresh weight ratios of the roots of P. nigra seedlings. The ratio of the root dry weight to fresh weight for block IV, treated with Simazine at 4 lbs per acre, is between 0.67 and 0.84, whilst the ratio of the root dry weight to root fresh weight for the remainder of the plots is between 0.30 and 0.20. This difference occurs irrespective of Simazine treatment.

The correlation coefficient between fresh weight and dry weight for this plot (block IV treated with 4 lbs Simazine per acre) is appreciably higher (0.89) than the equivalent correlation coefficient for the Kennington experimental plots as a whole (0.61).

Analysis of variance and calculation of the standard deviations showed that the dry weights of seedlings taken from the Kennington plots which had received 1, 2, or 4 lbs Simazine *per* acre varied significantly for any one treatment. No significant difference was found for the dry weights of seedlings grown on plots which had received 0 or 8 lbs Simazine per acre (Table 9).

Further analysis of variance and calculations of standard deviations for the other variates measured showed that the observed differences between dry weight and fresh weight ratios for each treatment were of limited significance and did not statistically affect the other characters of the root system.

In the light of this result, it was decided to consider problems of site effects in an experiment set up at Bedford College, the results of which are given in Tables 13 and 14.

Table 9. Analysis of variance of root dry weights of two year old P.nigra seedlings sampled in Kennington Forest plot 456FY70.

Lbs. Simazine per acre applied	Block	Mean	Standard Deviation	Degrees of Freedom	F.value	Significance
0	I	1.2509	0.4877	11		
	II	1.1420	0.5699	10		
	III	1.1900	0.3043	12		
	IV	1.2618	0.4863	22		
				3/51	0.1922	Not significant
1	I	1.1284	0.4669	13		
	III	0.9393	0.3869	15		
	IV	1.5700	0.5682	25		
				2/50	8.3197	0.1%
2	I	1.3233	0.4479	12		
	II	1.1241	0.4222	12		
	III	1.2500	0.3423	18		
	IV	0.8376	0.3869	21		
				3/59	5.2748	1.0%
4	I	1.1411	0.7996	9		
	II	1.1561	0.4638	13		
	III	1.3475	0.4972	12		
	IV	0.6416	0.3085	19		
				3/49	5.8947	1.0%
8	I	1.2858	0.4035	12		
	II	1.3275	0.6638	12		
	III	1.3627	0.4474	11		
	IV	1.1875	0.6847	20		
				3/51	0.2638	Not significant

The anomalous dry weight/fresh weight ratios observed in block IV 4 lbs Simazine per acre are reflected in the significant F. value obtained on analysis of variance of root dry weights.

a) The effect of Simazine on the mycorrhizas and uninfected root tips of *P.sylvestris* and *P.nigra* growing in forest plots 458FY70 (Kennington) and 83FY70 (Wareham).

(i) *P.sylvestris.*

In some cases soil supplied Simazine may have affected the numbers of mycorrhizal roots of *P.sylvestris* (see Tables, 11 and 12 and Figures 19, 20 and 21).

Mycorrhizas developing on seedlings sampled in November 1970 at the Kennington site increased after an application of 4 lbs /acre (Table 11). Seedlings sampled at the Wareham site in November 1970 showed a marked increase in the numbers of mycorrhizal roots after the application of Simazine. Mycorrhizas of seedlings sampled in November1972 at the same Wareham site did not respond to any applications of Simazine (Table 12).

Applications of Simazine on the same forest plots and seedlings affected the number of uninfected root tips per tree at one site. Seedlings growing on the Kennington plot to which applications at 4 lbs Simazine per acre had been made (sampled in November 1970) showed a clear increase in the numbers of uninfected root tips per tree (Table 11). No such effect of Simazine on the total number of uninfected root tips per tree (c.f. uninfected root tips per g) was observed in the Wareham site(when sampled in November 1970 and 1972 these seedlings growing on the Wareham site which had received Simazine

showed no apparent increase in numbers of uninfected short roots per tree (Table 12)).

(ii) P.nigra

Soil applied Simazine may affect the numbers of mycorrhizal roots of P.nigra in similar ways to P.sylvestris (see Tables 10 and 11 and Figures 19, 20 and 21).

In November 1970 the numbers of mycorrhizas per seedling tree (Kennington) were not affected by any applications of Simazine (Table 11). Seedlings sampled in November 1970 at the Wareham site (Table 10) which had received applications of Simazine, showed some increase in the numbers of mycorrhizas, especially with the application of 8 lbs Simazine per acre. However, in February 1972, on the same site and with the same applications of Simazine, the numbers of mycorrhizas developing on short roots were not affected by any of the applications of Simazine.

Simazine had little effect on the numbers of uninfected root tips of seedlings sampled in November 1970 in the Kennington and Wareham sites, and similarly, in February 1972, in the Wareham site (Tables 10 and 11).

Table 10. 1970 and 1972 assessments of the means and standard errors obtained for the experimental plot 83FY70 (Wareham), to show the effect of Simazine on the root systems of *Pinus nigra*.

November 1970 assessment	0 lbs/acre Simazine		1 lb/acre Simazine		2 lbs/acre Simazine		4 lbs/acre Simazine		8 lbs/acre Simazine	
Sample size (N)	55		53		63		53		55	
P. nigra	Mean	Std. error	Mean	Std. error	Mean	Std. error	Mean	Std. error	Mean	Std. error
Fresh weight of shoot (g)	-	-	-	-	-	-	-	-	-	-
Fresh weight of root (g)	3.18	(0.165)	3.41	(0.215)	2.41	(0.156)	2.25	(0.195)	3.13	(0.217)
No. of uninfected root tips/g root	95.68	(4.877)	88.38	(5.746)	140.94	(8.960)	131.65	(7.754)	105.41	(7.907)
No. of mycorrhizas/tree	13.95	(1.101)	17.13	(1.768)	19.43	(1.564)	16.72	(1.789)	25.13	(2.083)
No. of dichotomous roots/tree	36.11	(0.932)	22.06	(0.701)	16.52	(0.424)	10.81	(0.295)	18.00	(0.741)
No. of lateral roots/tree	-	-	-	-	-	-	-	-	-	-
No. of 'pioneer' roots/tree	-	-	-	-	-	-	-	-	-	-
No. of uninfected root tips/tree	286.96	(17.043)	294.66	(25.270)	290.93	(17.803)	268.06	(22.190)	333.62	(37.328)

February 1972 assessment	0 lbs/acre Simazine		1 lb/acre Simazine		2 lbs/acre Simazine		4 lbs/acre Simazine		8 lbs/acre Simazine	
Sample size (N)	82		100		100		100		83	
P. nigra	Mean	Std. error	Mean	Std. error	Mean	Std. error	Mean	Std. error	Mean	Std. error
Fresh weight of shoot (g)	15.41	(0.828)	12.64	(0.610)	16.11	(0.744)	15.72	(0.724)	12.66	(0.756)
Fresh weight of root (g)	4.42	(0.240)	2.97)	(0.156)	3.78	(0.222)	3.92	(0.217)	3.60	(0.290)
No. of uninfected root tips/g root	65.91	(4.550)	45.52	(2.677)	47.81	(2.465)	61.90	(2.799)	54.59	(6.124)
No. of mycorrhizas/tree	6.39	(1.485)	11.82	(4.567)	5.25	(1.422)	4.63	(1.086)	5.06	(1.315)
No. of dichotomous roots/tree	6.56	(0.848)	3.09	(0.437)	3.49	(0.426)	3.38	(0.384)	3.24	(0.574)
No. of lateral roots/tree	3.75	(0.386)	5.07	(0.251)	4.67	(0.637)	5.20	(0.645)	5.47	(0.453)
No. of 'pioneer' roots/tree	5.73	(0.375)	3.00	(0.208)	4.92	(0.392)	3.74	(0.283)	3.13	(0.382)
No. of uninfected root tips/tree	185.59	(10.550)	172.77	(11.233)	155.94	(9.748)	160.85	(9.741)	189.18	(12.618)

Table 11. 1970 assessments of the means and standard errors, obtained for the experimental plot 458FY70 (Kennington), to show the effect of Simazine on the root systems of *Pinus sylvestris* and *Pinus nigra*.

P. sylvestris	0 lbs/acre Simazine		1 lb/acre Simazine		2 lbs/acre Simazine		4 lbs/acre Simazine		8 lbs/acre Simazine	
Sample size (N)	53		53		49		43		52	
Nov. 1970 assessment	Mean	Std. error	Mean	Std. error	Mean	Std. error	Mean	Std. error	Mean	Std. error
Fresh weight of shoot(g)	-	-	-	-	-	-	-	-	-	-
Fresh weight of root(g)	3.33	(0.223)	4.66	(0.387)	3.40	(0.202)	6.03	(0.414)	3.72	(0.179)
No. of uninfected root tips/g root	69.92	(6.339)	98.45	(5.688)	87.02	(6.931)	107.01	(8.281)	86.30	(9.111)
No. of mycorrhizas/tree	29.53	(8.848)	28.58	(3.711)	16.40	(1.107)	46.34	(4.393)	21.30	(1.397)
No. of dichotomous roots/tree	26.98	(8.288)	26.72	(5.510)	21.61	(3.087)	36.51	(7.069)	23.67	(3.503)
No. of lateral roots/tree	-	-	-	-	-	-	-	-	-	-
No. of 'pioneer' roots/tree	-	-	-	-	-	-	-	-	-	-
No. of uninfected root tips/tree	332.71	(22.365)	465.79	(38.668)	340.34	(20.270)	602.69	(41.370)	371.78	(17.889)

P. nigra	0 lbs/acre Simazine		1 lb/acre Simazine		2 lbs/acre Simazine		4 lbs/acre Simazine		8 lbs/acre Simazine	
Sample size (N)	51		46		42		48		45	
Nov. 1970 assessment	Mean	Std. error	Mean	Std. error	Mean	Std. error	Mean	Std. error.	Mean	Std. error
Fresh weight of shoot(g)	-	-	-	-	-	-	-	-	-	-
Fresh weight of root (g)	3.40	(0.227)	3.34	(0.257)	2.55	(0.229)	3.92	(0.251)	2.77	(0.210)
No. of uninfected root tips/g root	78.20	(4.540)	100.51	(9.786)	107.86	(7.528)	56.68	(3.197)	106.27	(6.897)
No. of mycorrhizas/tree	20.92	(2.110)	21.32	(2.134)	20.40	(1.989)	15.22	(1.459)	18.80	(1.729)
No. of dichotomous roots/tree	5.94	(1.113)	4.02	(1.421)	2.142	(0.382)	7.23	(3.267)	4.11	(0.546)
No. of lateral roots/tree	-	-	-	-	-	-	-	-	-	-
No. of 'pioneer' roots/tree	-	-	-	-	-	-	-	-	-	-
No. of uninfected root tips/tree	269.68	(21.908)	272.50	(24.390)	240.64	(18.460)	213.35	(15.480)	271.11	(19.300)

Table 12. 1970 and 1972 assessments of the means and standard errors, obtained for the experimental plot 83FY70 (Wareham), to show the effect of Simazine on the root systems of *Pinus sylvestris*.

November 1970 assessment	0 lbs/acre Simazine		1 lb/acre Simazine		2 lbs/acre Simazine		4 lbs/acre Simazine		8 lbs/acre Simazine	
Sample size (N)	42		57		50		51		54	
P. sylvestris	Mean	Std. error	Mean	Std. error	Mean	Std. error	Mean	Std. error	Mean	Std. error
Fresh weight of shoot(g)	-	-	-	-	-	-	-	-	-	-
Fresh weight of root (g)	5.42	(0.372)	5.25	(0.299)	5.89	(0.380)	5.25	(0.429)	3.41	(0.286)
No. of uninfected root tips/g root	139.73	(8.005)	147.66	(8.004)	162.42	(8.840)	131.07	(14.920)	216.91	(15.720)
No. of mycorrhizas/tree	10.43	(10.778)	76.95	(9.822)	173.86	(26.310)	115.00	(37.156)	62.38	(10.310)
No. of dichotomous roots/tree	28.19	(3.345)	28.61	(4.203)	22.86	(2.005)	14.76	(2.298)	22.33	(2.400)
No. of lateral roots/tree	-	-	-	-	-	-	-	-	-	-
No. of 'pioneer' roots/tree	-	-	-	-	-	-	-	-	-	-
No. of uninfected root tips/tree	740.26	(81.630)	771.98	(64.620)	882.82	(48.600)	535.19	(43.095)	648.95	(45.053)

November 1972 assessment	0 lbs/acre Simazine		1 lb/acre Simazine		2 lbs/acre Simazine		4 lbs/acre Simazine		8 lbs/acre Simazine	
Sample size (N)	67		72		68		66		73	
P. sylvestris	Mean	Std. error	Mean	Std. error	Mean	Std. error	Mean	Std. error	Mean	Std. error
Fresh weight of shoot(g)	9.06	(0.340)	11.46	(0.288)	9.74	(0.327)	8.12	(0.338)	11.03	(0.355)
Fresh weight of root (g)	5.97	(0.270)	6.54	(0.196)	6.21	(0.226)	5.39	(0.247)	7.39	(0.246)
No. of uninfected root tips/g root	99.00	(9.431)	102.46	(6.999)	103.00	(6.470)	90.96	(7.973)	100.69	(6.595)
No. of mycorrhizas/tree	8.91	(2.436)	12.75	(2.726)	8.01	(2.456)	14.47	(3.316)	12.55	(2.701)
No. of dichotomous roots/tree	2.57	(0.813)	6.41	(1.457)	2.74	(0.647)	4.66	(0.856)	8.92	(2.091)
No. of lateral roots/tree	6.24	(0.270)	5.66	(0.195)	5.75	(0.226)	5.68	(0.247)	6.35	(0.246)
No. of 'pioneer' roots/tree	1.31	(0.116)	1.43	(0.110)	1.10	(0.114)	1.27	(0.117)	0.77	(0.115)
No. of uninfected root tips/tree	515.86	(52.554)	640.56	(58.499)	610.30	(59.700)	492.53	(52.710)	670.62	(56.308)

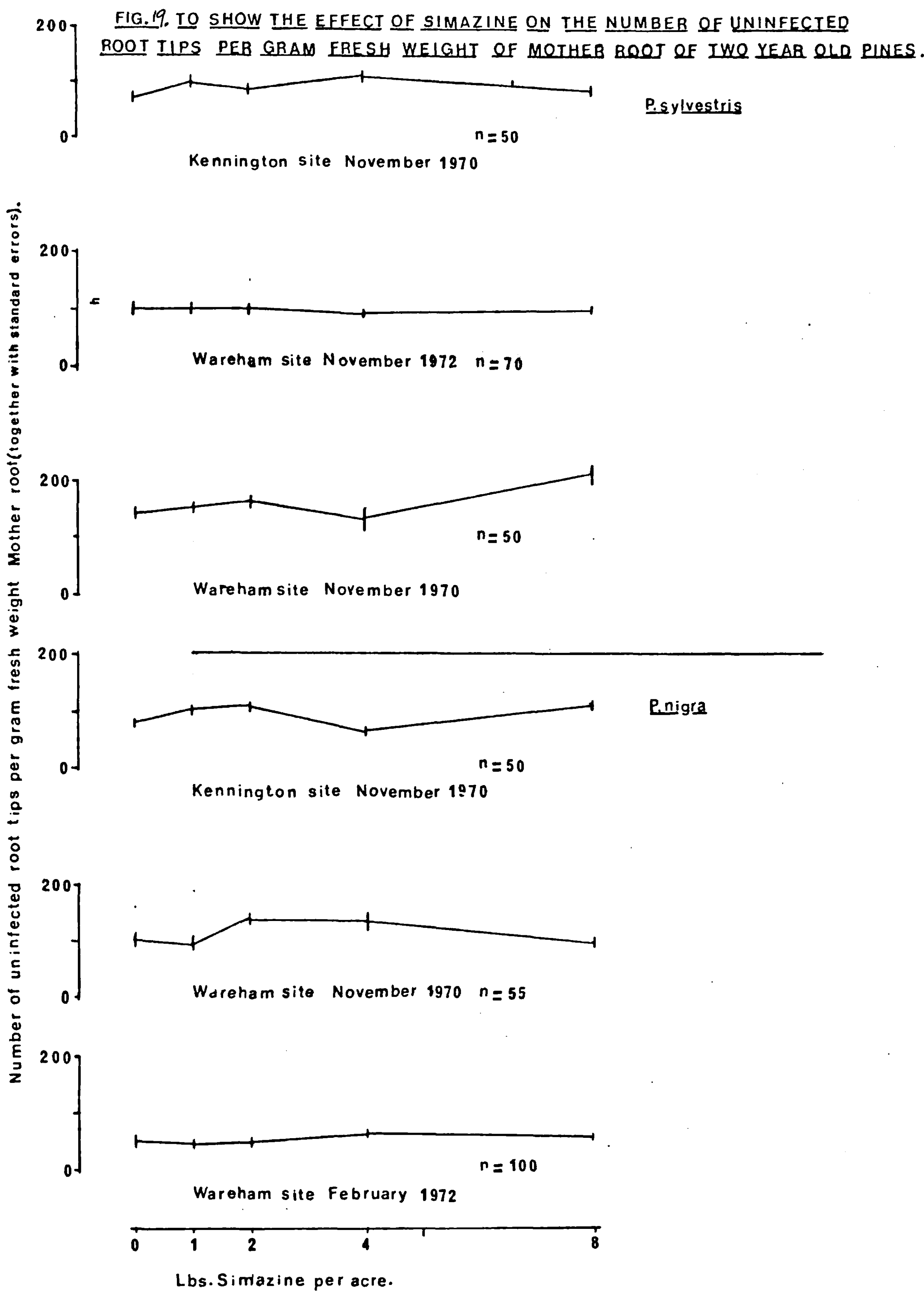
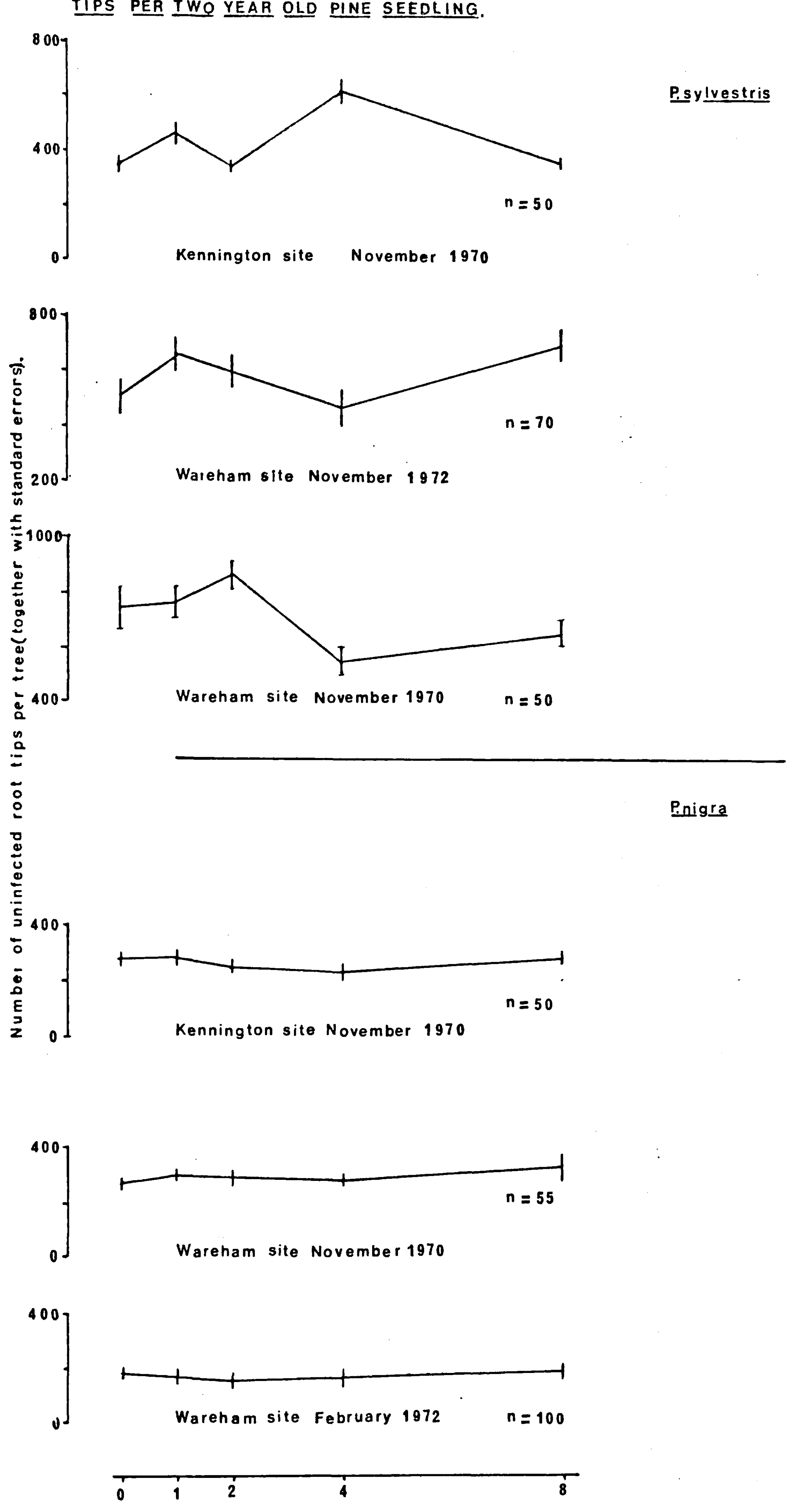


FIG. 19. TO SHOW THE EFFECT OF SIMAZINE ON THE NUMBER OF UNINFECTED ROOT TIPS PER GRAM FRESH WEIGHT OF MOTHER ROOT OF TWO YEAR OLD PINES.
P. sylvestris
P. nigra
Number of uninfected root tips per gram fresh weight Mother root (together with standard errors).
200
0
n = 50
Kennington site November 1970
Wareham site November 1972 n = 70
n = 50
Wareham site November 1970
n = 50
Kennington site November 1970
Wareham site November 1970 n = 55
n = 100
Wareham site February 1972
0
1
2
4
8
Lbs. Simazine per acre.

FIG. 20. TO SHOW THE EFFECT OF SIMAZINE ON THE NUMBER OF UNINFECTED ROOT TIPS PER TWO YEAR OLD PINE SEEDLING.



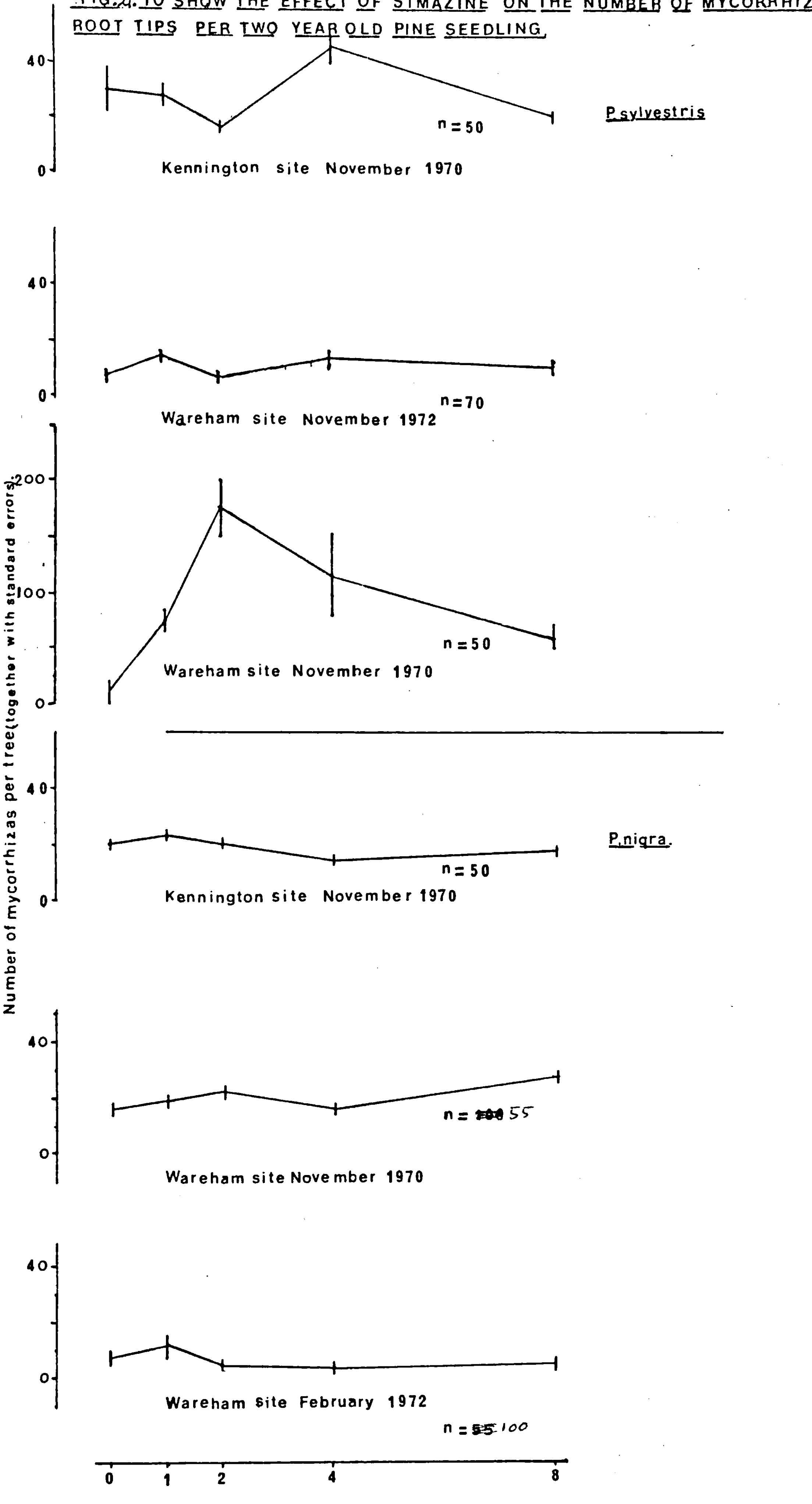


FIG.21. TO SHOW THE EFFECT OF SIMAZINE ON THE NUMBER OF MYCORRHIZAL ROOT TIPS PER TWO YEAR OLD PINE SEEDLING.
P.sylvestris
n = 50
Kennington site November 1970
n=70
Wareham site November 1972
n = 50
Wareham site November 1970
P.nigra.
n = 50
Kennington site November 1970
n = 55
Wareham site November 1970
Wareham site February 1972
n = 100
Number of mycorrhizas per tree(together with standard errors)
0
40
100
200
0
1
2
4
8
Lbs. Simazine per acre.

b) The effects of Simazine on the mycorrhizas of P. sylvestris and P. nigra seedlings growing in plant pots containing soil taken from Thetford Chase (chalky soil), Kennington (silty loam) or Wareham (sandy soil).

The Pine root systems were assessed 18 months after potting; the soils did not contain EHC, fertiliser supplements, or supplementary organic material. Roots were more common around the plant pot side and coralloid mycorrhizas confined to the roots growing at the edge of the plant pot. However, dichotomous mycorrhizal roots were distributed throughout the root system.

Due to the high level of uninfected root tips, assessment was difficult. For this reason the numbers of uninfected root tips/g fresh weight 'mother' root were counted instead of the numbers of uninfected root tips per seedling.

Certain general effects on the pine root systems were noted; the effects varied with differences in soil type, and with the application of Simazine. P. sylvestris seedlings grown in Thetford soils showed an increase in the number of lateral roots (Table 13).

This increase in the numbers of lateral roots consisted of an increase in both 'mother' and subordinate 'mother' roots and was not observed in any other instance.

P.sylvestris and P.nigra seedlings growing in plant pots containing the various soils from Wareham or Thetford treated with 4 lbs / acre Simazine generally showed more mycorrhizas than the untreated controls(Tables 13 and 14).

The number of non-mycorrhizal, dichotomous root tips per tree were always very low and were not affected by the application of 4 lbs / acre Simazine. However, P.sylvestris and P.nigra growing in sandy Wareham soil treated with 4 lbs /acre Simazine showed an increase in the number of uninfected short roots per gram fresh weight (Tables 13 and 14).

Table 13. The means and standard errors obtained from the experiment to show the effects of Simazine on the root systems of P. sylvestris growing in plant pots containing different soil types.

P. sylvestris growing in soil from Thetford Chase.

	0 lbs/acre Simazine		4 lbs/acre Simazine	
Sample size (N)	44		33	
	Mean	Std. error	Mean	Std. error
Fresh weight of shoot (g)	22.43	(1.705)	25.44	(1.604)
Fresh weight of root (g)	14.67	(1.090)	19.27	(1.241)
No. of uninfected root tips/g fresh weight†	110.86	(9.072)	127.78	(9.456)
No. of mycorrhizas/tree	178.30	(30.309)	241.55	(48.010)
No. of dichotomous roots/tree*	2.34	(0.591)	1.12	(0.369)
No. of lateral roots/tree	9.50	(0.750)	15.79	(1.640)
No. of pioneer roots/tree	2.87	(0.688)	3.39	(1.123)

P. sylvestris growing in soil from Wareham

Sample size (N)	26		15	
Fresh weight of shoot (g)	23.88	(2.608)	22.15	(1.207)
Fresh weight of root (g)	17.32	(1.509)	18.13	(1.128)
No. of uninfected root tips/g fresh weight†	101.73	(7.099)	173.80	(8.866)
No. of mycorrhizas/tree	274.11	(34.400)	501.40	(39.408)
No. of dichotomous roots/tree*	4.58	(1.364)	11.87	(2.877)
No. of lateral roots/tree	12.35	(1.021)	14.27	(1.089)
No. of pioneer roots/tree	1.92	(0.432)	2.13	(0.601)

P. sylvestris growing in soil from Kennington

Sample size	18		16	
Fresh weight of shoot (g)	19.57	(1.470)	19.71	(2.947)
Fresh weight of root (g)	14.18	(1.337)	14.86	(2.382)
No. of uninfected root tips/g fresh weight†	92.17	(6.789)	72.94	(9.860)
No. of mycorrhizas/tree	55.61	(10.469)	89.38	(21.020)
No. of dichotomous roots/tree*	0.11	(0.076)	0.0	(0.000)
No. of lateral roots/tree	9.72	(1.035)	9.62	(1.408)
No. of pioneer roots/tree	2.22	(0.482)	1.06	(0.348)

* Dichotomous roots are non-mycorrhizal.

† 'mother' root.

Table 14. The means and standard errors obtained from the experiment to show the effects of Simazine on the root systems of P.nigra growing in plant pots containing different soil types.

P.nigra growing in soil from Thetford Chase.

	0 lbs/acre Simazine		4 lbs/acre Simazine	
Sample size (N)	12		11	
	Mean	Std. error	Mean	Std. error
Fresh weight of shoot (g)	16.18	(2.084)	13.02	(2.523)
Fresh weight of root (g)	9.88	(1.227)	8.50	(1.614)
No. of uninfected root tips/g fresh weight†	36.50	(3.059)	14.18	(8.229)
No. of mycorrhizas/tree	10.92	(4.560)	32.36	(8.925)
No. of dichotomous roots/tree*	0.0	(0.000)	0.0	(0.000)
No. of lateral roots/tree	7.42	(0.721)	5.63	(1.029)
No. of pioneer roots/tree	1.56	(0.743)	1.36	(0.717)

P.nigra growing in soil from Wareham.

Sample size (N)	16		20	
Fresh weight of shoot (g)	10.56	(2.640)	19.15	(1.747)
Fresh weight of root (g)	7.30	(1.506)	15.15	(1.308)
No. of uninfected root tips/g fresh weight†	23.56	(4.297)	76.55	(4.615)
No. of mycorrhizas/tree	26.94	(6.274)	51.45	(4.562)
No. of dichotomous roots/tree*	0.19	(0.188)	0.10	(0.100)
No. of lateral roots/tree	4.69	(1.106)	7.70	(0.837)
No. of pioneer roots/tree	1.00	(0.303)	2.95	(1.859)

P.nigra growing in soil from Kennington

Sample size (N)	17		19	
Fresh weight of shoot (g)	16.01	(1.384)	17.14	(2.429)
Fresh weight of root (g)	10.11	(0.676)	10.10	(1.508)
No. of uninfected root tips/g fresh weight†	74.71	(5.699)	67.22	(6.753)
No. of mycorrhizas/tree	28.43	(5.883)	56.78	(12.334)
No. of dichotomous roots/tree*	0.0	(0.000)	0.11	(0.076)
No. of lateral roots/tree	10.85	(0.733)	7.89	(0.963)
No. of pioneer roots/tree	1.29	(0.270)	1.22	(0.223)

* Dichotomous roots are non mycorrhizal.

† 'mother root'.

3. THE HERBICIDE EFFECT ON THE IN VITRO GROWTH OF FUNGAL ISOLATES.

Radial growth rates were compared on basic ion agar and are given in Table 15.

Table 15. The growth rates of fungal isolates on basic ion agar plates (mm/wk) at 25°

Isolate	Rate	Isolate	Rate
B. granulatus	25.0	C. graniforme	16.0
B. luteus	26.5	R. emetica	20.0
B. variegatus	17.0	A. rubescens	18.5
B. badius	15.0	T. terrestris	17.5
B. bovinus	15.5	A. mellea	15.5
B. edulis	9.5	M.R. atrovirens	19.0
B. elegans	19.0	P. graminis	10.5
F. annosus	20.0		

These growth rates were obtained from measurement of isolates in their linear growth phase.

a) Herbicide effect assessed by agar plates.

A plot of colony growth on basic ion agar containing known concentrations of relevant herbicide, with respect to time, gave a curve with three consecutive phases:

1. Little but accelerating growth (lag and acceleration phase).
2. Rapid and approximately linear growth.
3. Little or no net growth (approaching stationary phase).

The inhibitory or stimulatory effect of the herbicide on the growth rate of the fungal isolate is expressed as the percentage difference between the linear growth rate on plates containing known concentrations of herbicide, and the linear growth rate of the control. However, the fundamental criticism of the technique, that is measurement of colony diameter instead of colony dry weight, was not met and for this reason some liquid culture assays were undertaken. With shake liquid culture assays it was not possible to carry out sufficient variety of treatments and adequate replication, hence agar plates were the main assay technique employed. Herbicide concentrations resulting in inhibitions at 70, 50 and 30 per cent levels of the controls are derived from Figures 22 to 27.

Several of the isolates inhibited by relatively low concentrations of herbicide showed little increase of inhibition at higher concentrations of herbicide; for example A.rubescens and M.R. atrovirens with Chlorthiamid (Figure 13).

Simazine and Atrazine did not inhibit any of the fungal isolates tested (Figures 26 and 27). Indeed some isolates appeared to be stimulated by certain concentrations of Simazine; A.mellea and B.granulatus were stimulated at concentrations of 0.4 and 0.5 ppm respectively; when the Simazine concentration was increased to 0.6 ppm the growth rates of these two isolates equalled those of the controls.

The isolates most inhibited by Dichlorobenzoate were B.bovinus (8 ppm), B.granulatus (8 ppm), B.badius (8 ppm) and C.graniforme (15 ppm); at these concentrations of herbicide, growth of these isolates was inhibited by 50 per cent relative to the control (Figure 22).

B.luteus (0.5 ppm), B.bovinus (0.8 ppm), B.elegans (1.5 ppm) and A.rubescens (2.0 ppm) were the isolates most inhibited by Dichlorobenzonitrile; at these concentrations of Dichlorobenzonitrile the isolateswere inhibited by 50 per cent relative to the control (Figure 24).

The isolates most inhibited by Chlorthiamid were C.graniforme (12.0 ppm), B.granulatus (15.0 ppm), B.variegatus (30.0 ppm); at these concentrations of Chlorothiamid the isolates are inhibited by 50 per cent relative to the control (Figure 23).

With 245T the isolates were inhibited by very much the same concentration range, a similar 50 per cent inhibition (relative to the control) occurring between 15 and 17 ppm 245T. Isolates of B.granulatus, A.mellea and P.graminis were exceptions; these showed 50 per cent inhibition (relative to the control) at 25 - 28 ppm 245T (Figure 25).

FIG.22 .

THE EFFECT OF DICHLOROBENZOATE ON THE RELATIVE GROWTH RATE (R.G.R.) OF MYCORRHIZAL FUNGI.

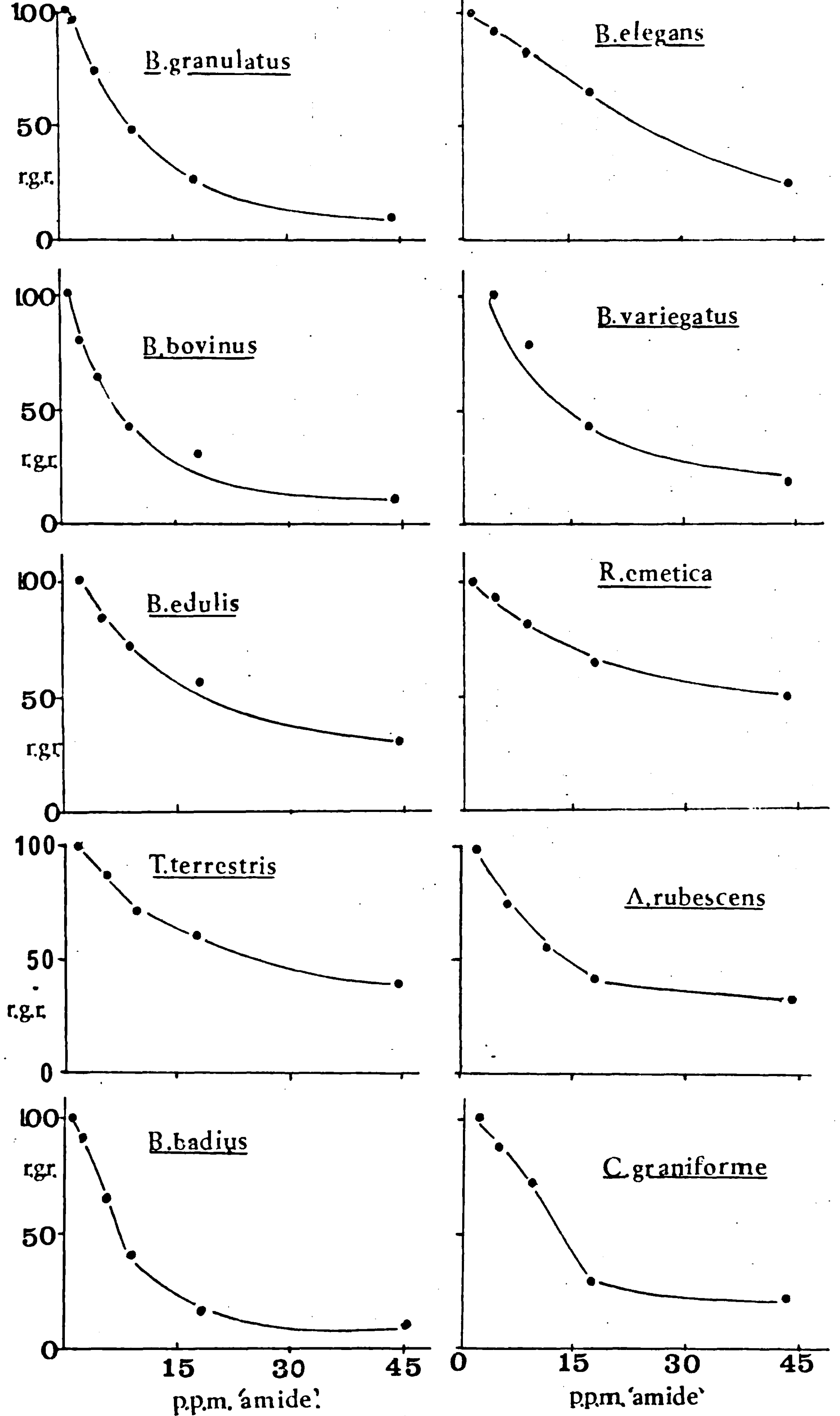
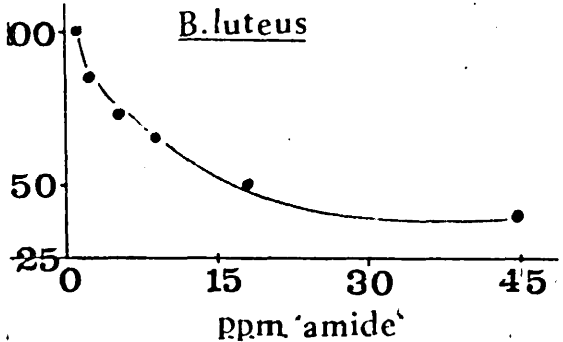


FIG.22.(cont).



THE EFFECT OF DICHLOROBENZOATE ON THE RELATIVE GROWTH RATE(RGR) OF PATHOGENIC ROOT FUNGI.

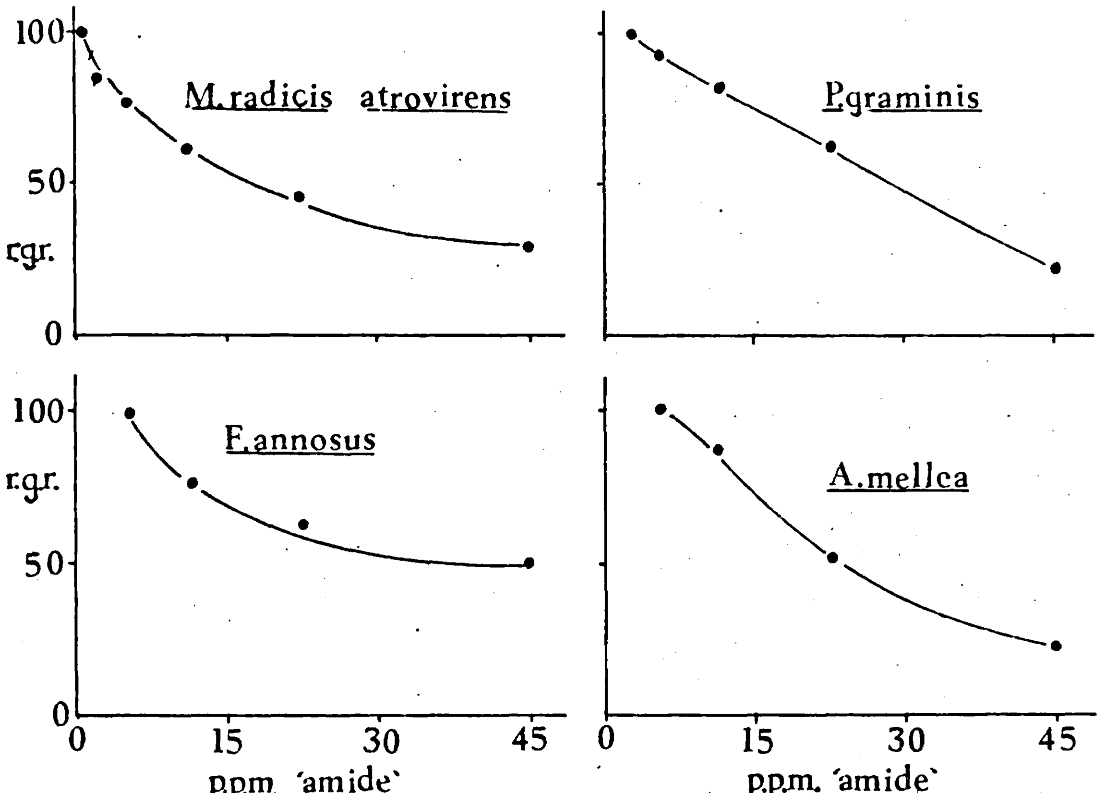


Fig.23 .

The effect of Prefix on the relative growth rate (r.g.r.) of mycorrhizal fungi.

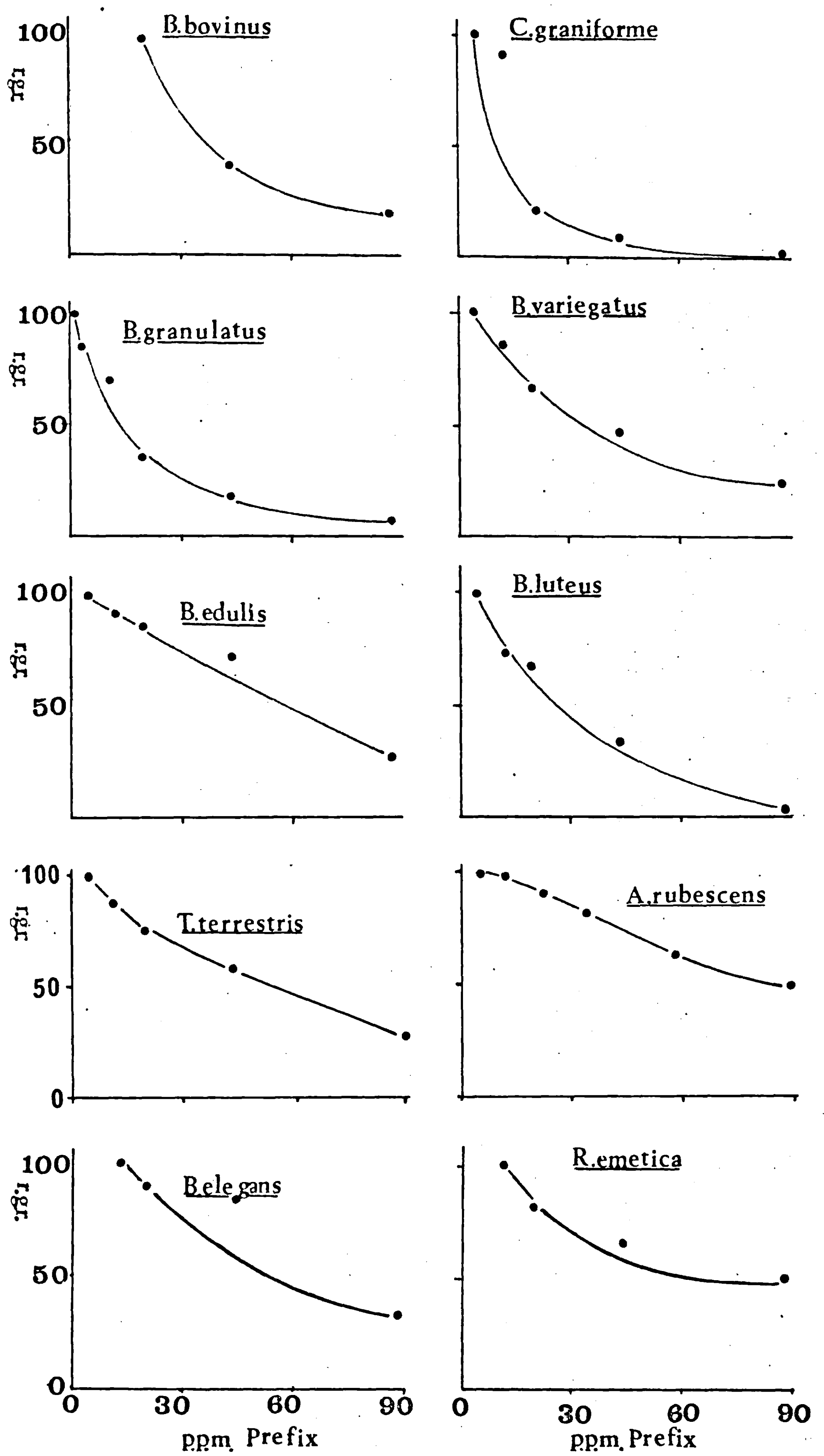


FIG. 23. (cont).

The effect of Prefix on the relative growth rate (r.g.r.) of pathogenic root fungi.

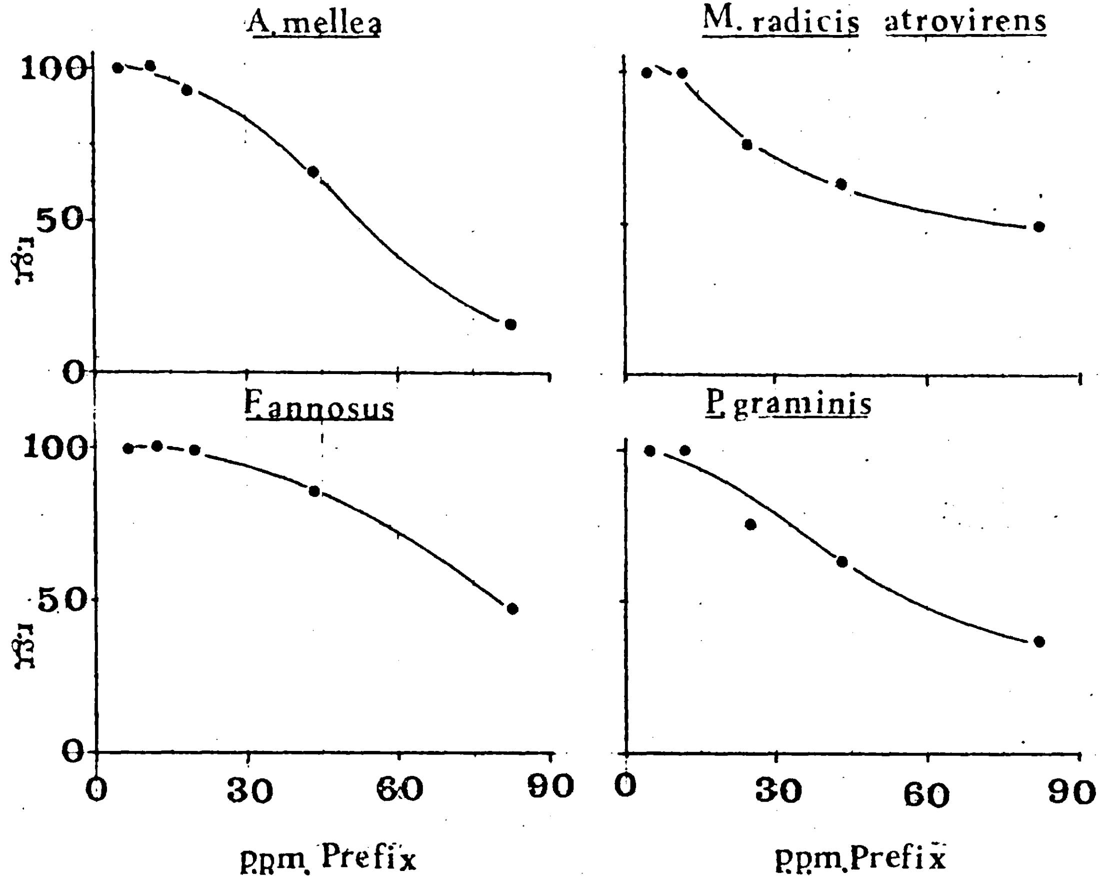


FIG. 24.

THE EFFECT OF DICHLOROBENZONITRILE ON THE RELATIVE GROWTH RATE (R.G.R.) OF MYCORRHIZAL FUNGI.

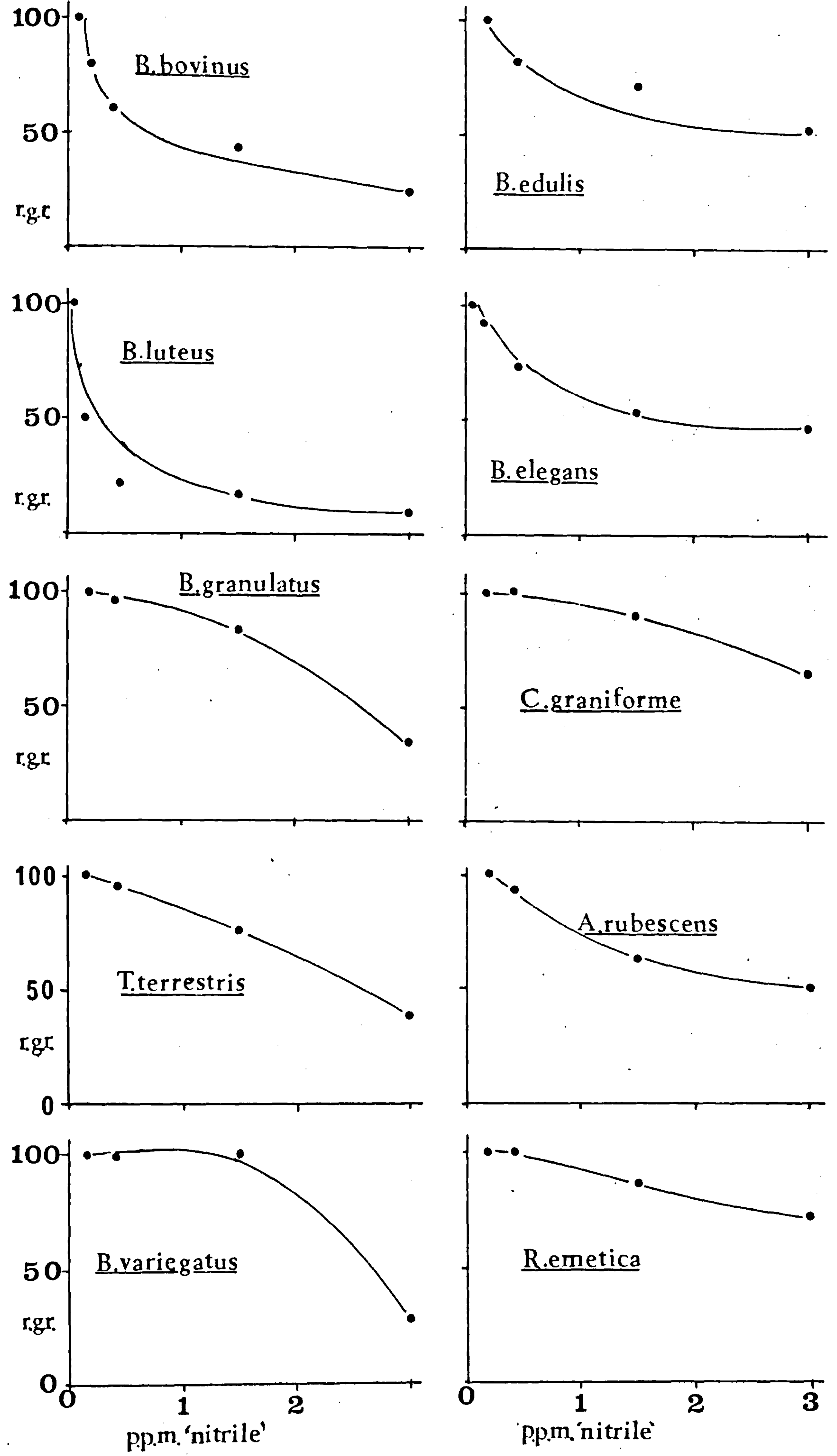


FIG. 24.(cont).

THE EFFECT OF DICHLOROBENZONITRILE ON THE RELATIVE GROWTH RATE(RGR) OF PATHOGENIC ROOT FUNGI.

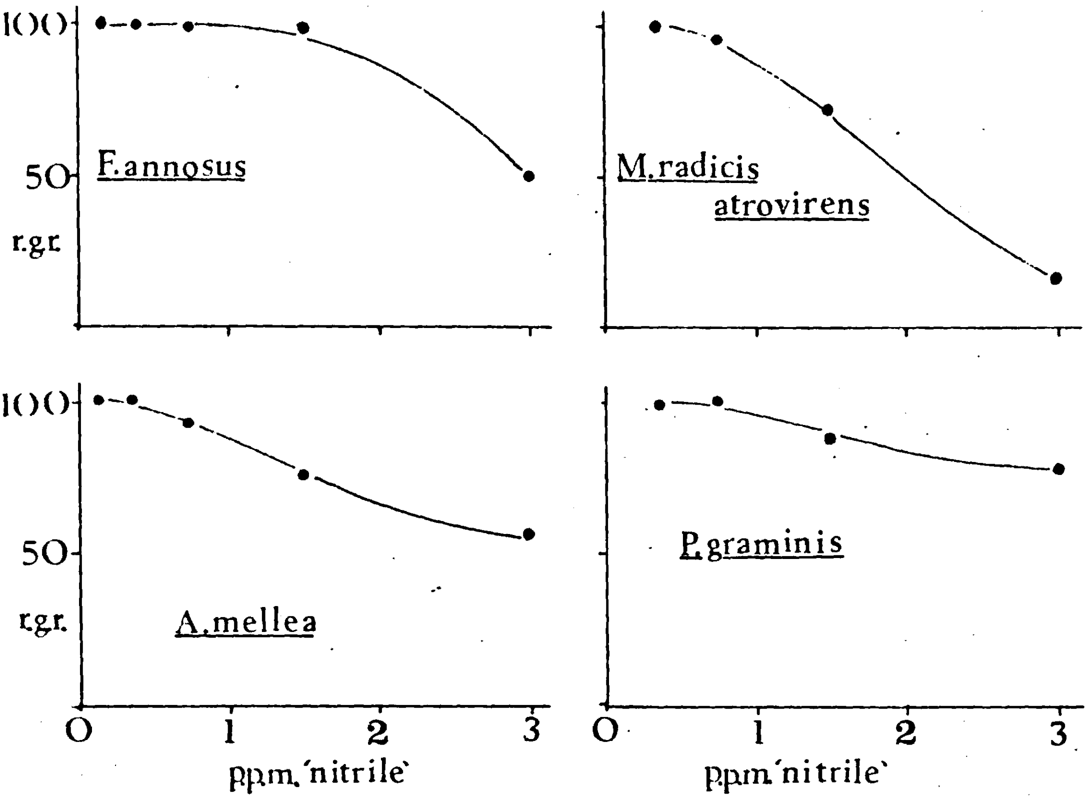


FIG. 25.

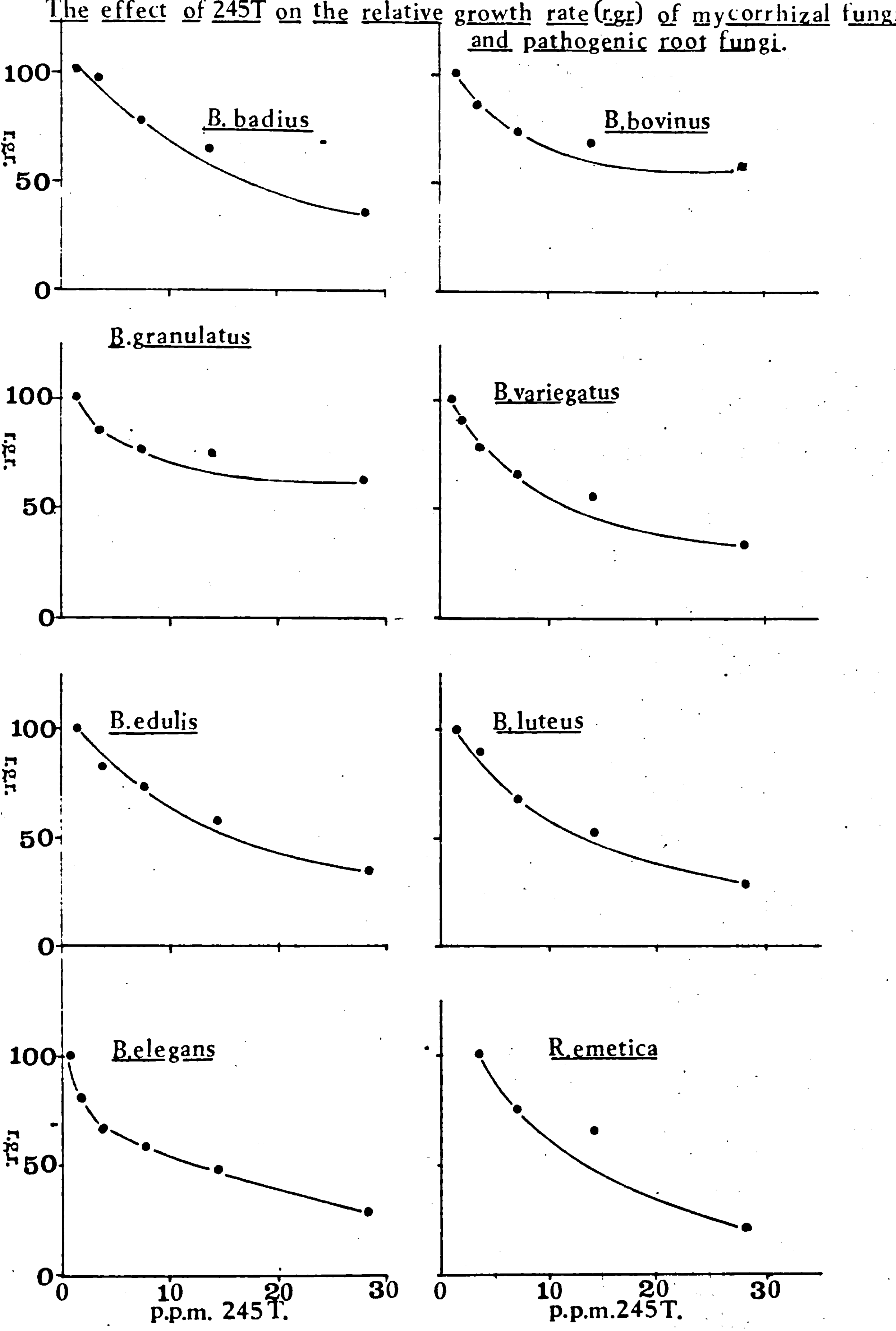


FIG. 25. (cont).

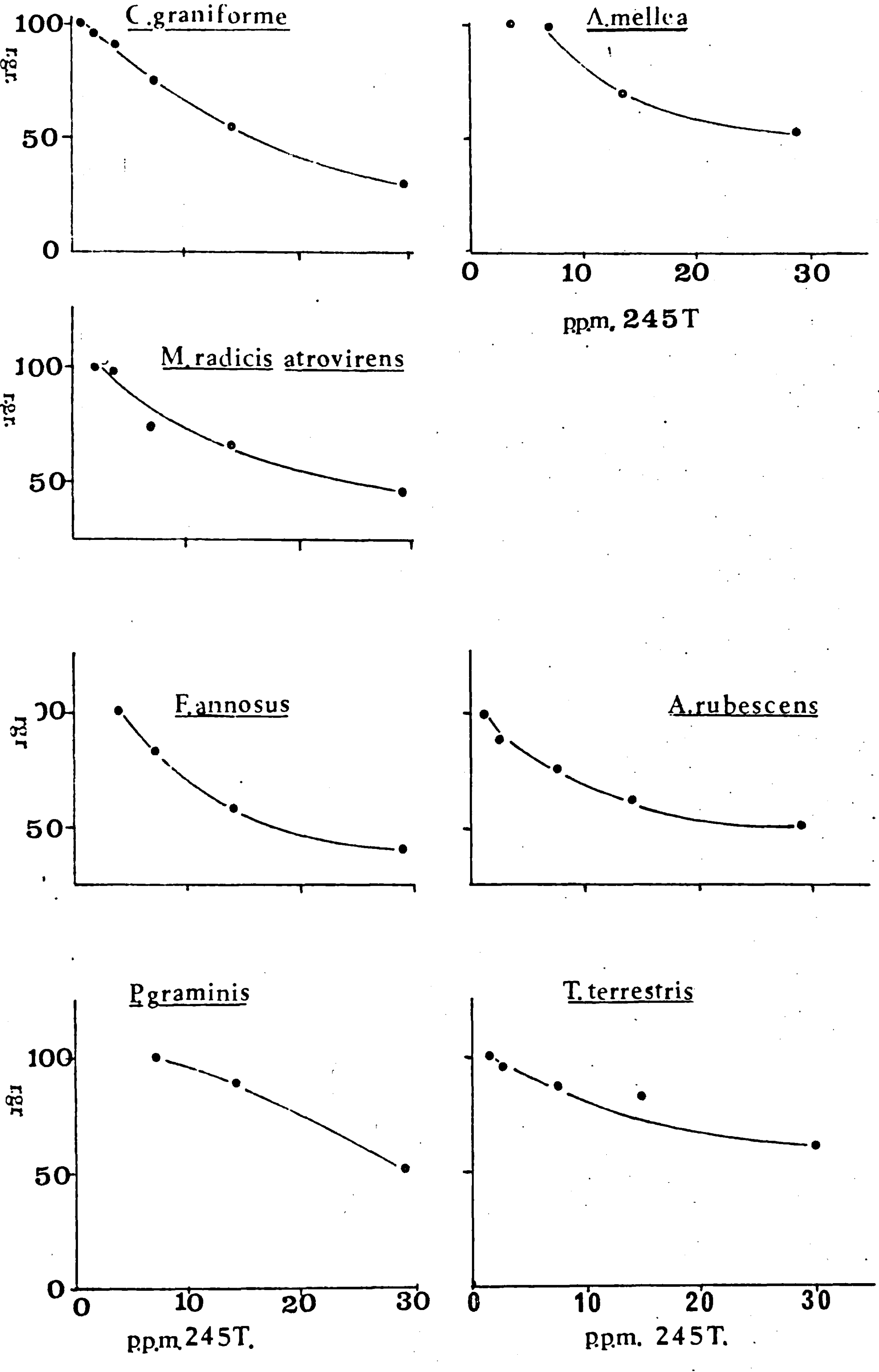


FIG. 26.

THE EFFECT OF SIMAZINE ON THE RELATIVE GROWTH RATE (R.G.R.) OF MYCORRHIZAL FUNGI.

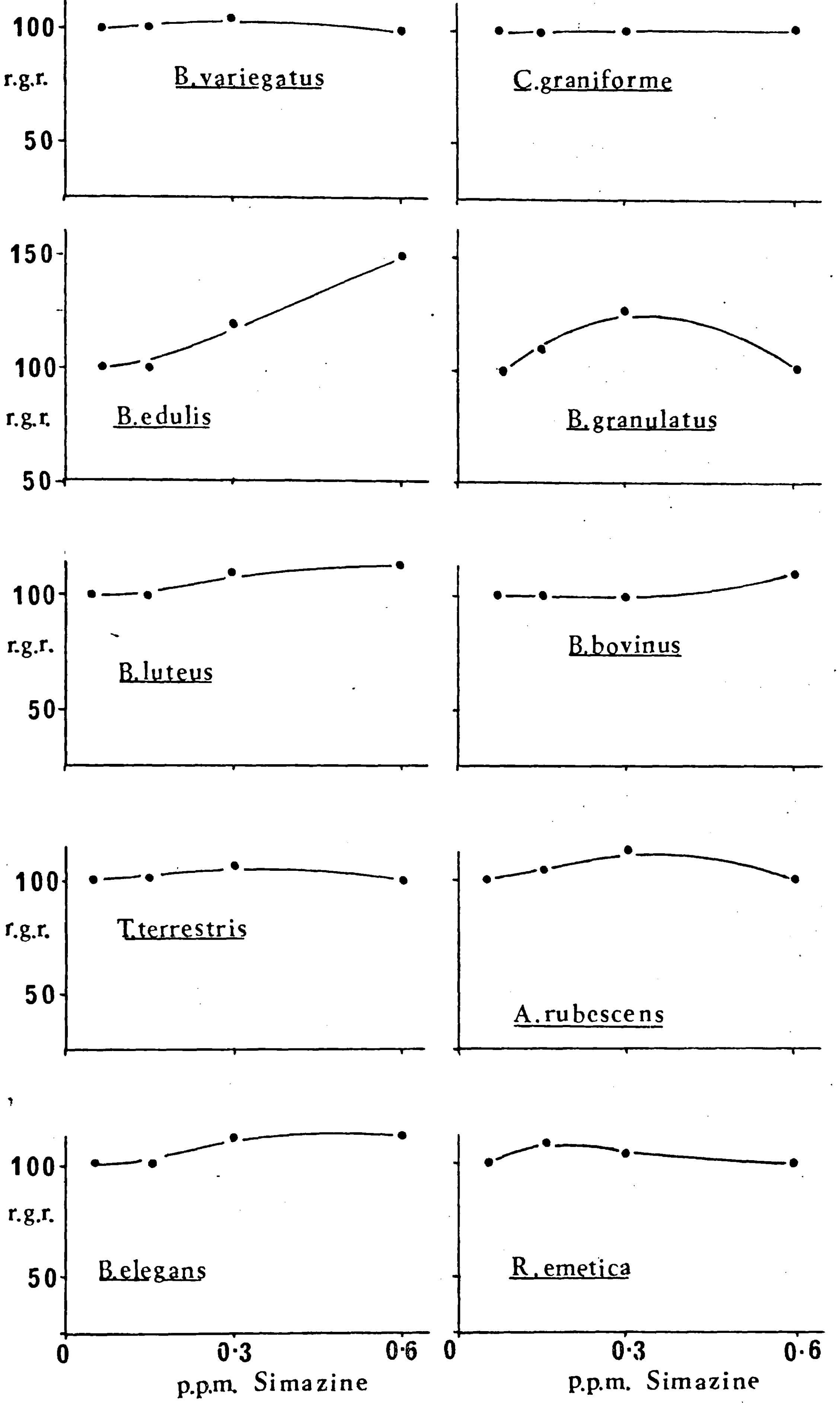


FIG.26.(cont).

THE EFFECT OF SIMAZINE ON THE RELATIVE GROWTH RATE (R.G.R.) OF PATHOGENIC ROOT FUNGI.

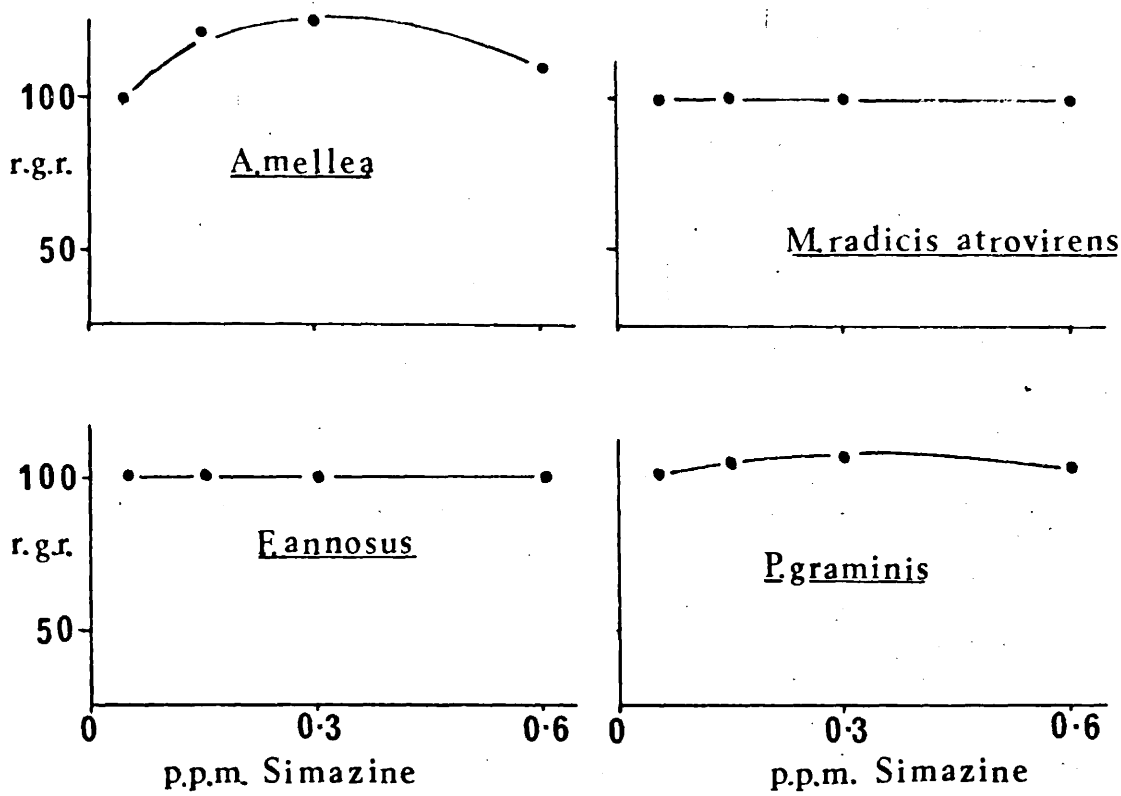


FIG.27.

THE EFFECT OF ATRAZINE ON THE RELATIVE GROWTH RATE (RGR) OF MYCORRHIZAL FUNGI.

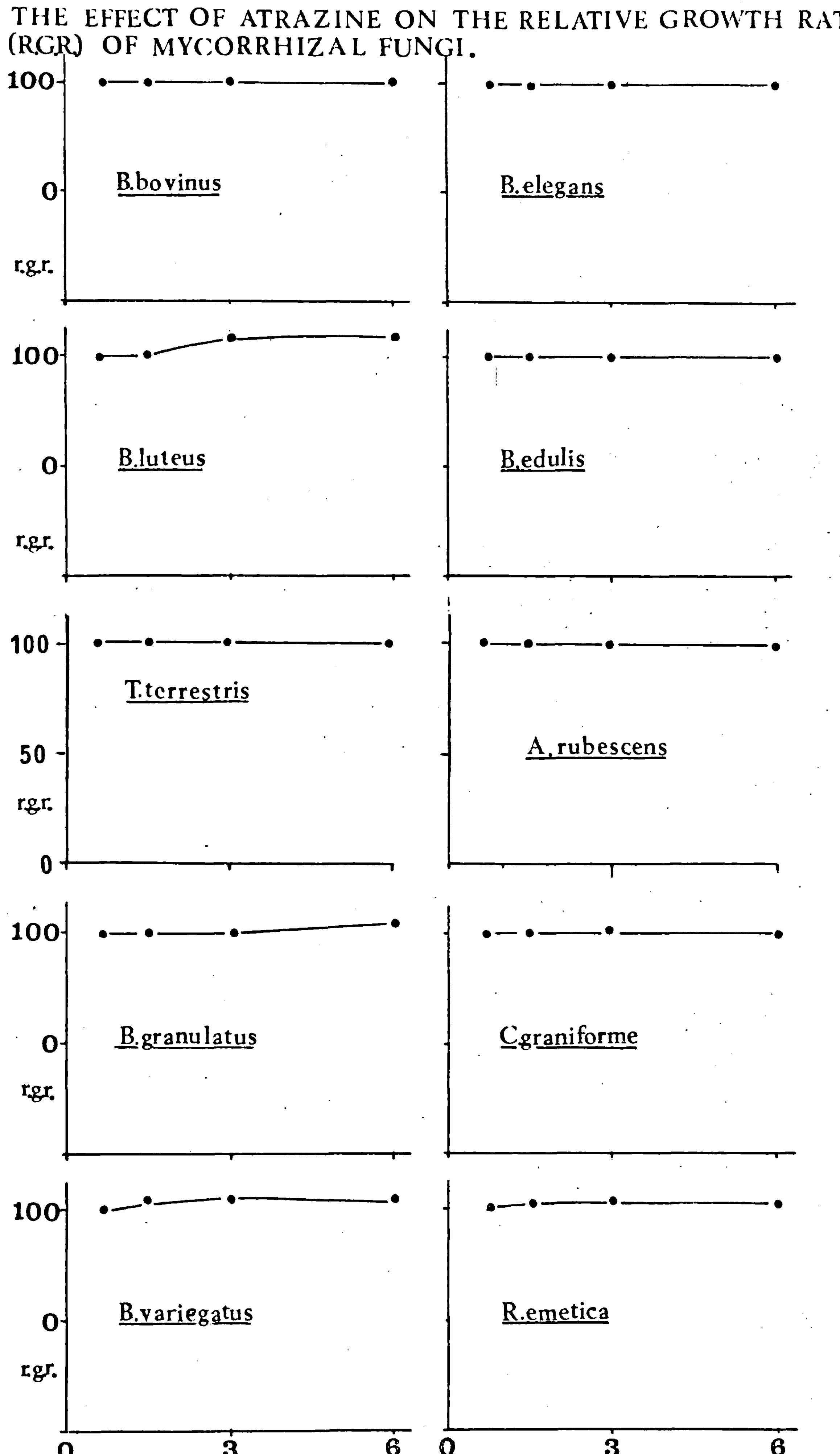
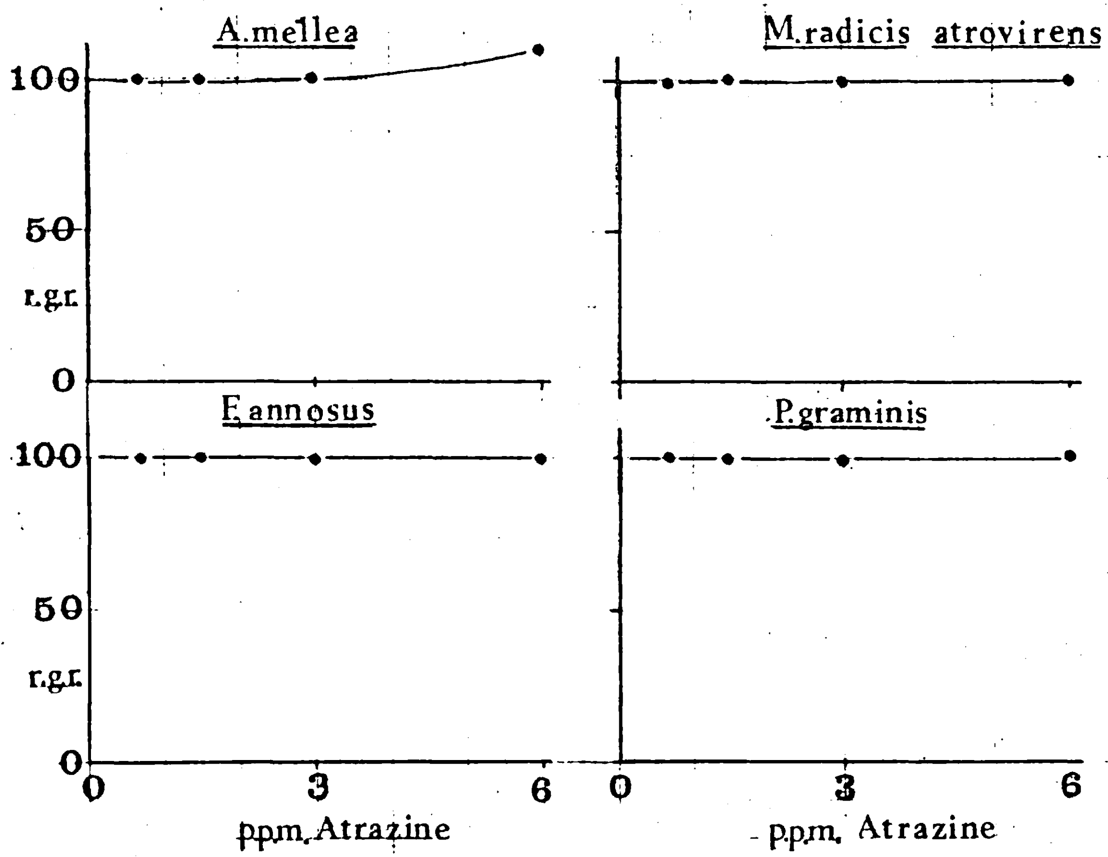


FIG.27.(cont).

THE EFFECT OF ATRAZINE ON THE RELATIVE GROWTH RATE (R.G.R.) OF PATHOGENIC ROOT FUNGI.



b) Herbicide effect assessed by shake liquid cultures.

Shake liquid cultures were used to confirm certain experimental results relating to the effect of herbicides on the growth of isolates on agar plates (Figures 28, 29 and 30). The concentrations known to inhibit the growth of the isolate on agar plates also affected the growth of the same isolates in shake liquid culture. The inoculum for shake liquid cultures, consisting of stripped and quartered 5mm plugs, is inevitably variable even though all precautions are taken to keep the inoculum as consistent as possible. Some inocula grown in shake liquid culture produce either fragmented mycelia or small compact pellets which subsequently fragment, these fragments in turn producing more compact small pellets. Other inocula growing in the absence of added herbicide or in only low herbicide concentrations developed (for any one set of conditions) into uniform mycelial pellets of reproducible dry weight. However, higher herbicide concentrations often produced erratic results. The use of 245T concentrations of 15 - 25 ppm inhibited all isolates (Figure 28). Chlorthiamid at concentrations of 8 - 15 ppm reduced the mycelial dry weight to 70% of the controls (Figure 30) for all isolates except R. emetica. Atrazine had no inhibitory effect on Boletus variegatus or Fomes annosus.

Other herbicides, for example Dichlorobenzonitrile and Dichlorobenzoate, known to inhibit growth of fungal isolates on agar plates also inhibited growth of these fungi in liquid culture. Hence the shake liquid culture results substantiated those obtained from the agar plate method. However, as a technique for accurately assaying herbicide effects on the fungal isolates the method is less satisfactory than agar plate assays for reasons mentioned previously.

FIG. 28.

THE EFFECT OF 245T ON THE RELATIVE GROWTH RATES OF MYCORRHIZAL AND PATHOGENIC ROOT FUNGI GROWN AS SHAKE LIQUID CULTURES.

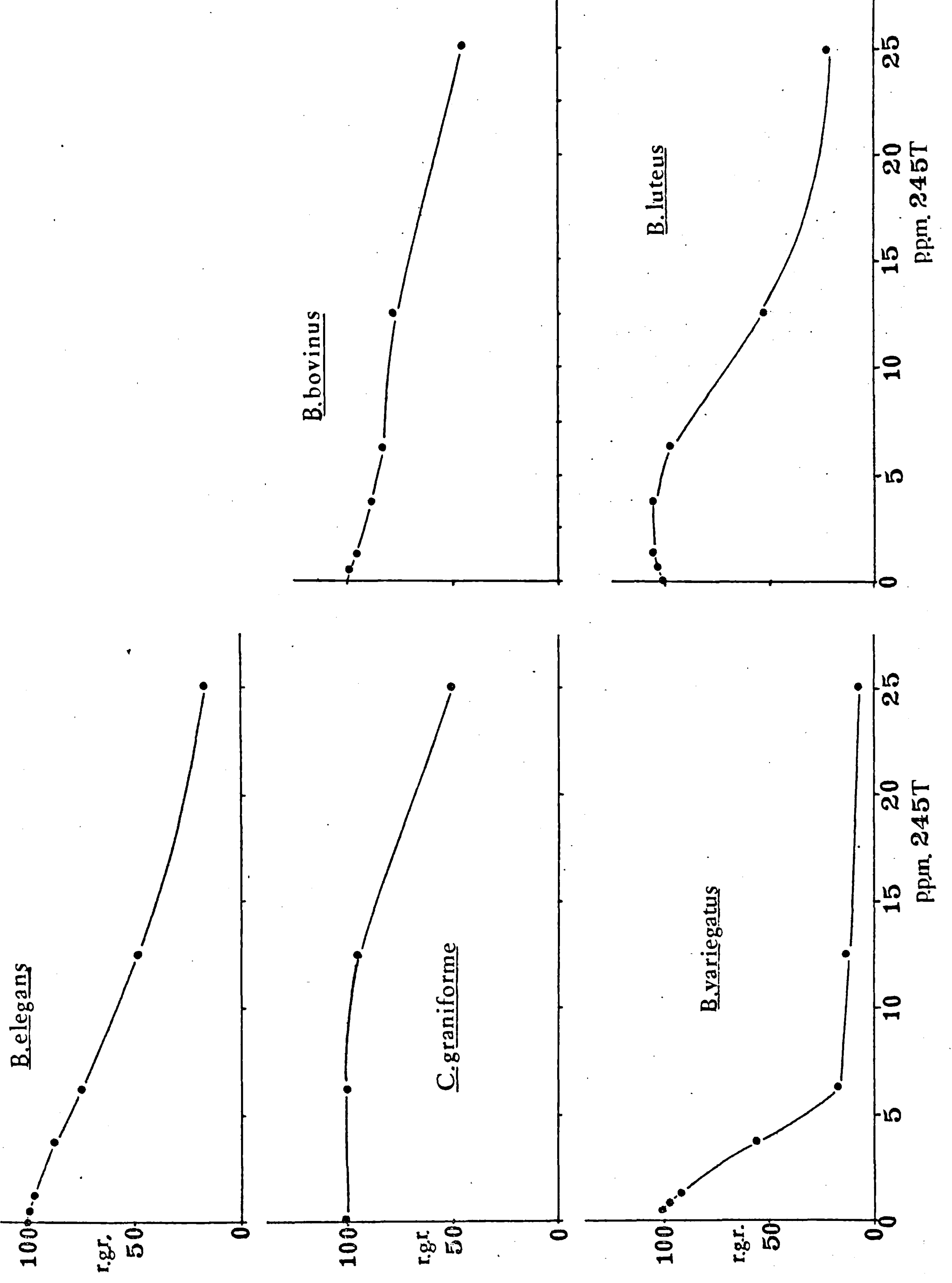


FIG. 29.

THE EFFECT OF ATRAZINE ON THE RELATIVE GROWTH RATES OF MYCORRHIZAL AND PATHOGENIC ROOT FUNGI GROWN AS SHAKE LIQUID CULTURES.

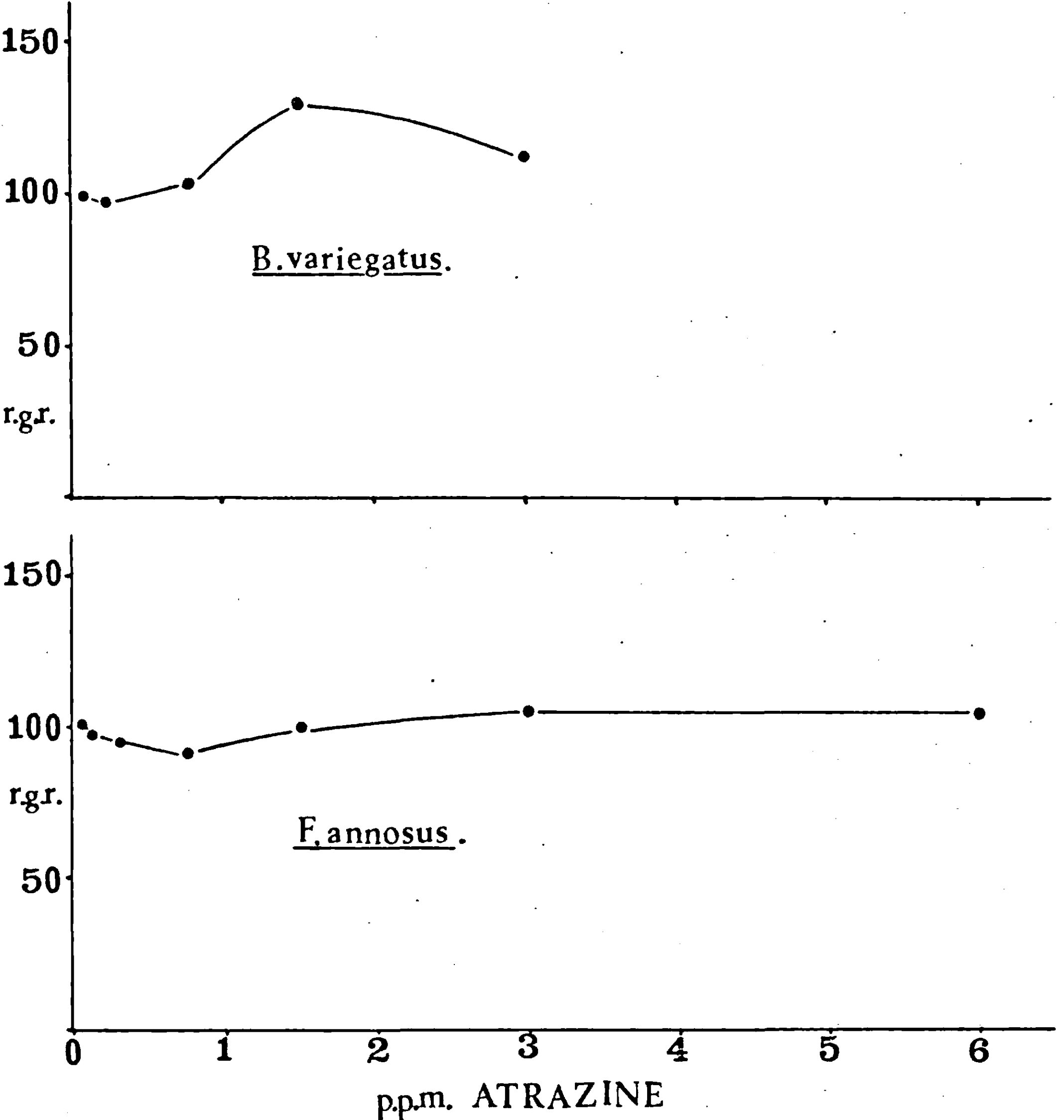
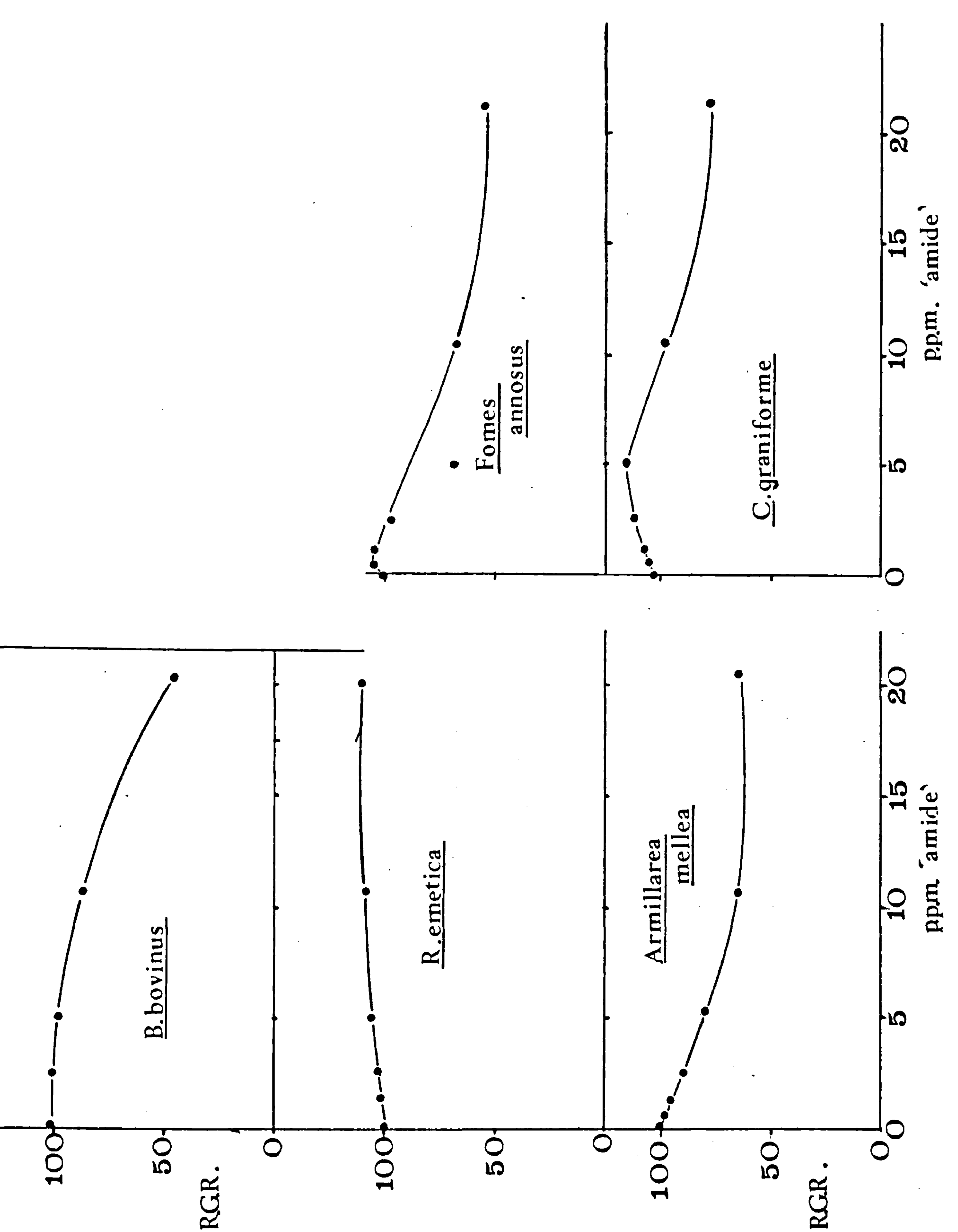


FIG. 30

THE EFFECT OF CHLORTHIAMID ON THE RELATIVE GROWTH RATE OF MYCORRHIZAL AND PATHOGENIC ROOT FUNGI GROWN AS SHAKE LIQUID CULTURES.



5. THE SYNTHESIS OF MYCORRHIZAS.

Melin's (1921), Fortin's (1966) and Trappe's (1967b) techniques for the synthesis of mycorrhizas were used with various degrees of success. With Trappe's technique, mycorrhizas were formed but reproducible high rates of success were not obtained. Certain changes in the composition of the media (see Table6), for example the reduction of glucose from 5 to either 0.1 or 1.0 g/l, coupled with a decrease of thiamin from 50 to 0.05 mg/l and a change in the pH from 4.5 to 3.0 caused changes in tree seedling growth but had no effect on the final numbers of mycorrhizas formed. Vermiculite containing Trappe's media or the media modified as above, inoculated with mycorrhizas fungus macerates, showed abundant mycelial growth and vigorous root systems. However, the pH of the media rose to about pH 7.0 after 60 - 70 days incubation. Sand containing any media was found to be unsuitable for root and mycelial growth, due to waterlogging and, paradoxically, drying out of the sand surface due to poor capillarity. The fungal isolates tested were the ones used in the Melin technique (see table 16).

Melin's technique was also found to be unsatisfactory. The number of mycorrhizas formed were low (Figures 31 and 32) even though the inoculum often grew as a mycelial lawn on the surface of the vermiculite and was often seen to colonise root surfaces. Some inocula, encouraged by the very high humidity in the flasks, grew up the shoots of the pine seedlings, and in one case an isolate resembling C. graniforme grew over the whole shoot, the vermiculite surface, and the root (Table 16).

Table 16. The combinations of fungi, inocula and media used in Melin's (1921) technique of mycorrhizal synthesis.

Seed	Fungal Isolate	Nature of inoculum	Incubation time	Nutrient medium	Root Matrix	Final pH of medium	Result
P. sylvestris	Mycelium radicis atrovirens	Agar plug	20 weeks	Melin's medium	Vermiculite	4.5	Trees killed
P. sylvestris	Russula emetica	Agar plug	20 weeks	Melin's medium	Vermiculite	4.5	Few mycorrhizas
P. sylvestris	Boletus bovinus	Agar plug	20 weeks	Melin's medium	Vermiculite	4.5	Mycorrhizas absent
P. sylvestris	Thelephora terrestris	Macerate	10 weeks	Sterile water	Vermiculite	5.5	Few mycorrhizas
P. nigra	Thelephora terrestris	Macerate	12 weeks	Sterile water	Perlite	5.5	Mycorrhizas absent
P. sylvestris	Amanita rubescens	Macerate	10 weeks	Sterile water	Perlite	5.5	Few mycorrhizas
P. nigra	Amanita rubescens	Macerate	10 weeks	Sterile water	Perlite	5.5	Mycorrhizas absent
P. sylvestris	Cenococcum graniforme? Isolate from Wareham roots	Agar plug	20 weeks	Sterile water	Vermiculite	4.5	Trees killed
P. sylvestris	Boletus variegatus	Agar plug	20 weeks	Melin's medium	Vermiculite	4.5	Few mycorrhizas

Figure 31.
Mycorrhizas synthesised using the technique of Melin (1921): A. rubescens on the host, P. sylvestris x2.

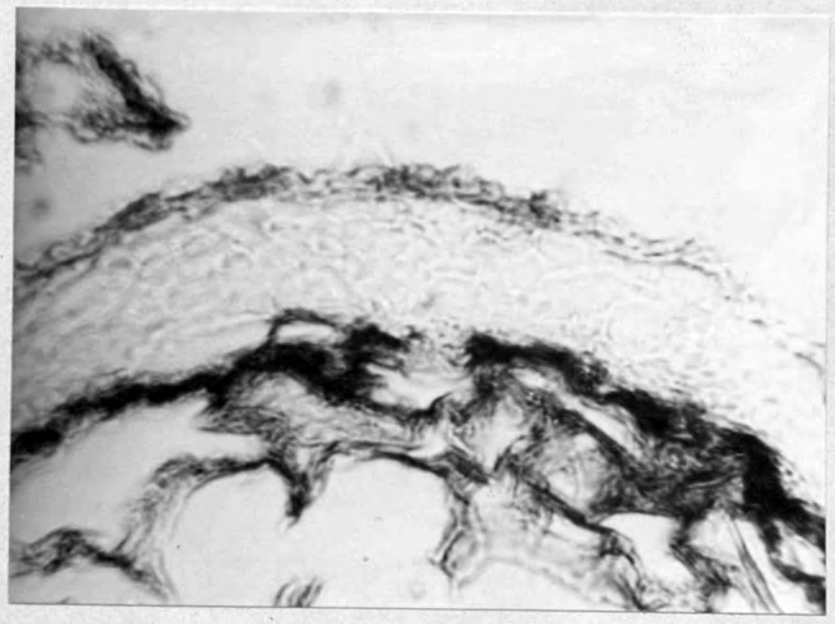


Figure 32.
Transverse section of a mycorrhiza from Figure 31. (x1000).

Fortin's technique was found to be more successful; about 32 - 38 per cent of the inoculated roots surviving the incubation produced mycorrhizas. This compares with Fortin's success rate of approximately 40 per cent. However, the percentage success of mycorrhizal formation with Fortin's technique was not large enough to obtain sufficient mycorrhizas for experiment. Very large numbers of 'explants' had to be set up and this was beyond the scope of the facilities and time available. It is apparent that the Fortin technique is successful; the choice of fungal isolate and the choice of media, though critical, are not necessarily the only factors which restrict the success rate for mycorrhizal syntheses. By careful control of temperature, media, pH, inoculum and growth periods it was possible to assess the effect of these variables on the root system. This enabled isolates to be characterised as to their specific effects, including mycorrhizal formation, on the root systems (Table 17; Figure 33).

The assessment of the pine roots inoculated with T.terrestris, A.rubescens, and B.variegatus is presented in Table 17. In all cases there was a recorded pH change of the Vermiculite from 4.5 to 7.0 after 60 - 70 days incubation. Mycorrhizas were formed using inocula of T.terrestris or A.rubescens on the excised roots of P.sylvestris. Success rates for mycorrhizal synthesis by the two inocula were 32 and 38 per cent respectively. The isolate of C.graniforme produced no mycorrhizas, but increased the total root length and slightly reduced the total number of lateral roots.

The small compact dichotomous mycorrhizal root tips formed in the above syntheses showed on examination a typical sheath with a

well defined Hartig net. Some transverse sections of the Hartig net showed that intra- as well as inter-cellular hyphae were present; these mycorrhizas were possibly of the ectendotrophic type (Wilcox, 1964) (Figure 31).

Table 17. The effects of fungal isolates on *P. sylvestris* root "explants", grown using the method of Fortin (1966).

	Thelephora terrestris		*Amanita rubescens*		*Boletus variegatus*		*Cenococcum graniforme*	
Sample size (N)	40		71		95		81	
	Mean	Std. error	Mean	Std. error	Mean	Std. error	Mean	Std. error error
Final pH of matrix	6.0	(0.48)	6.1	(0.42)	5.9	(0.90)	Not available	
Root length	28.1	(2.05)	26.2	(1.04)	27.9	(0.92)	34.9	(0.90)
Hypocotyl length	8.4	(0.29)	8.3	(0.62)	7.7	(0.68)	Not available	
Number of lateral roots	1.8	(0.32)	1.9	(0.23)	1.9	(0.22)	1.2	(0.14)
Total root length	6.4	(1.32)	6.0	(0.93)	7.0	(1.05)	6.3	(1.04)
Number of mycorrhizas	1.7	(0.11)	1.9	(0.11)	0.0	(0.00)	0.0	(0.00)

Roots were assessed 60-70 days after inoculation.

FIG.33.

THE RELATIONSHIP BETWEEN ROOT LENGTH AND THE NUMBER OF LATERAL ROOTS OF P. SYLVESTRIS, GROWN USING THE TECHNIQUE OF FORTIN.

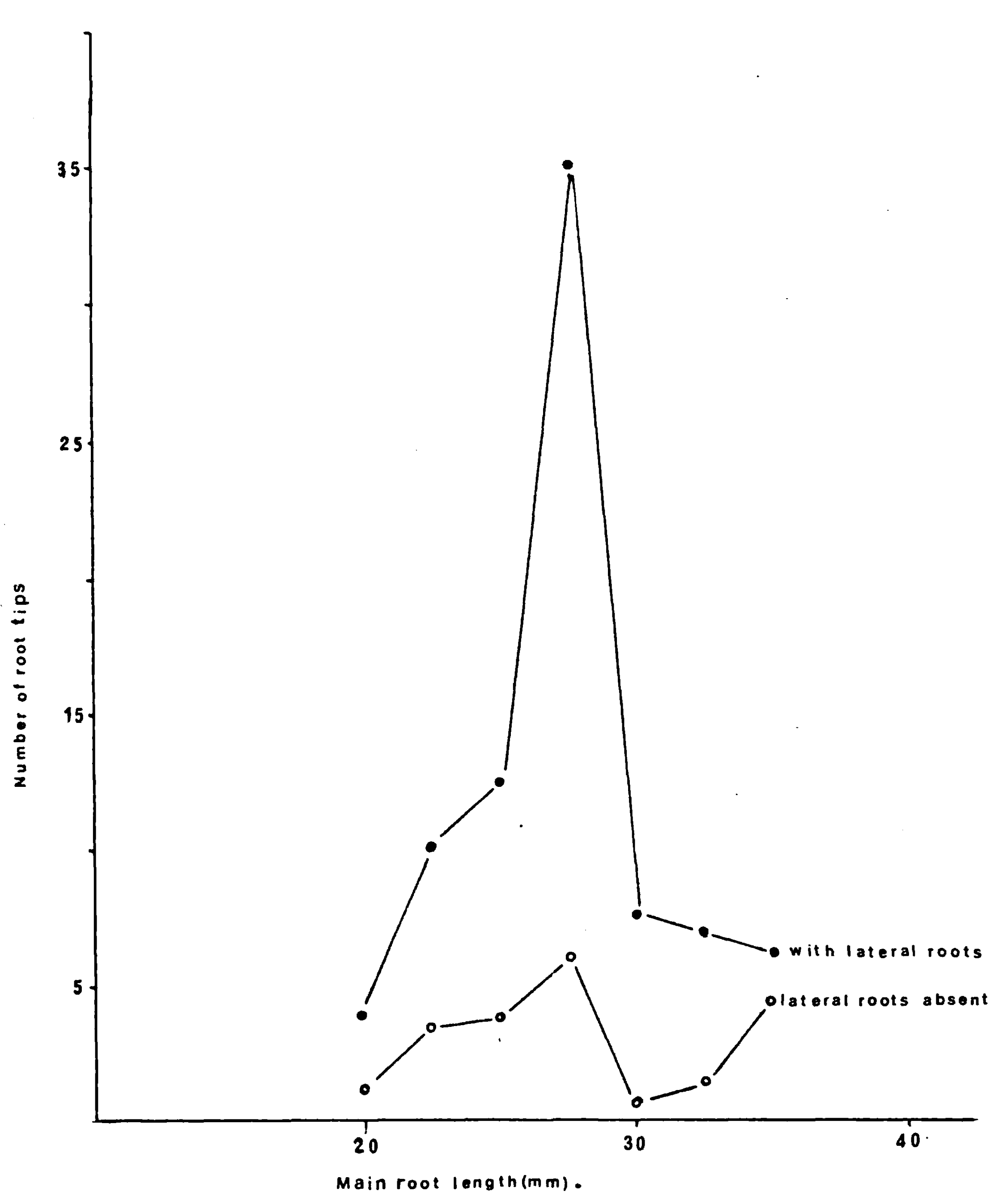


Figure 34
Coralloid mycorrhizas formed on "explants" of P. sylvestris inoculated with Thelephora terrestris. X2.

6. THE EFFECT OF HERBICIDES ON THE RESPIRATION RATES OF BOTH MYCORRHIZAL AND UNINFECTED ROOT TIPS OF P. SYLVESTRIS AND P. NIGRA.

The respiration rates of excised coralloid mycorrhizas were measured with an oxygen electrode. The morphology of these coralloid mycorrhizas corresponds in part to those mycorrhizas assessed for the effects of Simazine on the mycorrhizal roots of P. sylvestris and P. nigra growing in forest nursery plots and in plant pots (Fig. 4).

The roots of P. nigra possessed comparatively few well developed coralloid mycorrhizas and although sufficient coralloid mycorrhizas were collected to enable measurement of the effect of Atrazine on the respiration rate, the remainder of the experiments utilised the coralloid mycorrhizas of P. sylvestris.

The respiration rate of coralloid mycorrhizal roots of P. sylvestris sampled in March 1972 was less than half the respiration rate of similar roots sampled in September 1972 (0.49 and 1.12 $\mu l\ O_2$/minute/100 mg coralloid mycorrhizas respectively).

P. sylvestris and P. nigra, sampled in March 1972, had similar respiration rates (0.95 and 0.105 $\mu l\ O_2$/minute/100 mg uninfected root tips respectively). Coralloid mycorrhizal roots of P. nigra sampled in March 1972, gave respiration rates of 0.31 $\mu l\ O_2$/minute/100 mg coralloid mycorrhizal roots. Measurement of the respiration rates of coralloid mycorrhizas and uninfected roots of P. nigra was not undertaken in September 1972, due to lack of suitable mycorrhizal material.

Coralloid mycorrhizal roots of P. sylvestris, sampled in March 1972, pretreated with 30 ppm Atrazine or 4 ppm Simazine followed by ten minute recovery in fresh phthalate buffer, showed some increase in their respiration rates (see Figure 35). In September this increase in respiration rate was not noted after the application of either 30 ppm Atrazine or 4 ppm Simazine. The respiration rates of non mycorrhizal roots of P. sylvestris, sampled in March 1972, pretreated for ten minutes with 30 ppm Atrazine or 1 ppm Simazine showed stimulation; this was approximately 35 per cent over the control (Figure 35).

Non mycorrhizal short roots of P. nigra (sampled in March 1972) increased their respiration rate after ten minutes pretreatment with 0.5 ppm Atrazine. Coralloid mycorrhizal roots of P. nigra showed 100 per cent increase of respiration rate after ten minutes pretreatment with 2.5 ppm Atrazine.

Ten minute pretreatments with 245T at concentrations of 50 and 150 ppm respectively, followed by recovery in fresh phthalate buffer (ten minutes), reduced the respiration rates of coralloid mycorrhizas of P. sylvestris to 86 and 25 per cent of the control (Figure 36). Dichlobenil at low concentrations (5 ppm for ten minutes) may cause some stimulation in the respiration rate of coralloid mycorrhizas. However, at higher concentrations, pre-treatment with Dichlobenil (10 and 20 ppm) caused the respiration

rates of coralloid mycorrhizas to be reduced to 88 and 4 per cent of the controls(Figure 36). Ten minute pretreatments with Dichlobenzoate (Dichlorobenzoic acid) at 400 and 800 ppm caused respiration rates of coralloid mycorrhizas of P. sylvestris to be reduced to 80 and 5 per cent respectively (Figure 36). Although pretreatment with Chlorthiamid (Prefix) at low concentrations had no effect on the respiration rates of coralloid mycorrhizas of P. sylvestris, ten minute pretreatments with Chlorthiamid at 200 and 400 ppm caused respective reductions of the respiration rates to 75 and 50 per cent of the controls (Figure 36).

FIG. 35.

THE RESPIRATION RATES OF 100mg. SAMPLES OF MYCORRHIZAL AND UNINFECTED ROOT TIPS OF P. SYLVESTRIS AFTER 10 MINUTES PRETREATMENT WITH VARIOUS CONCENTRATIONS OF ATRAZINE OR SIMAZINE (TEMP = 20°C; TREES SAMPLED IN MARCH, 1971).

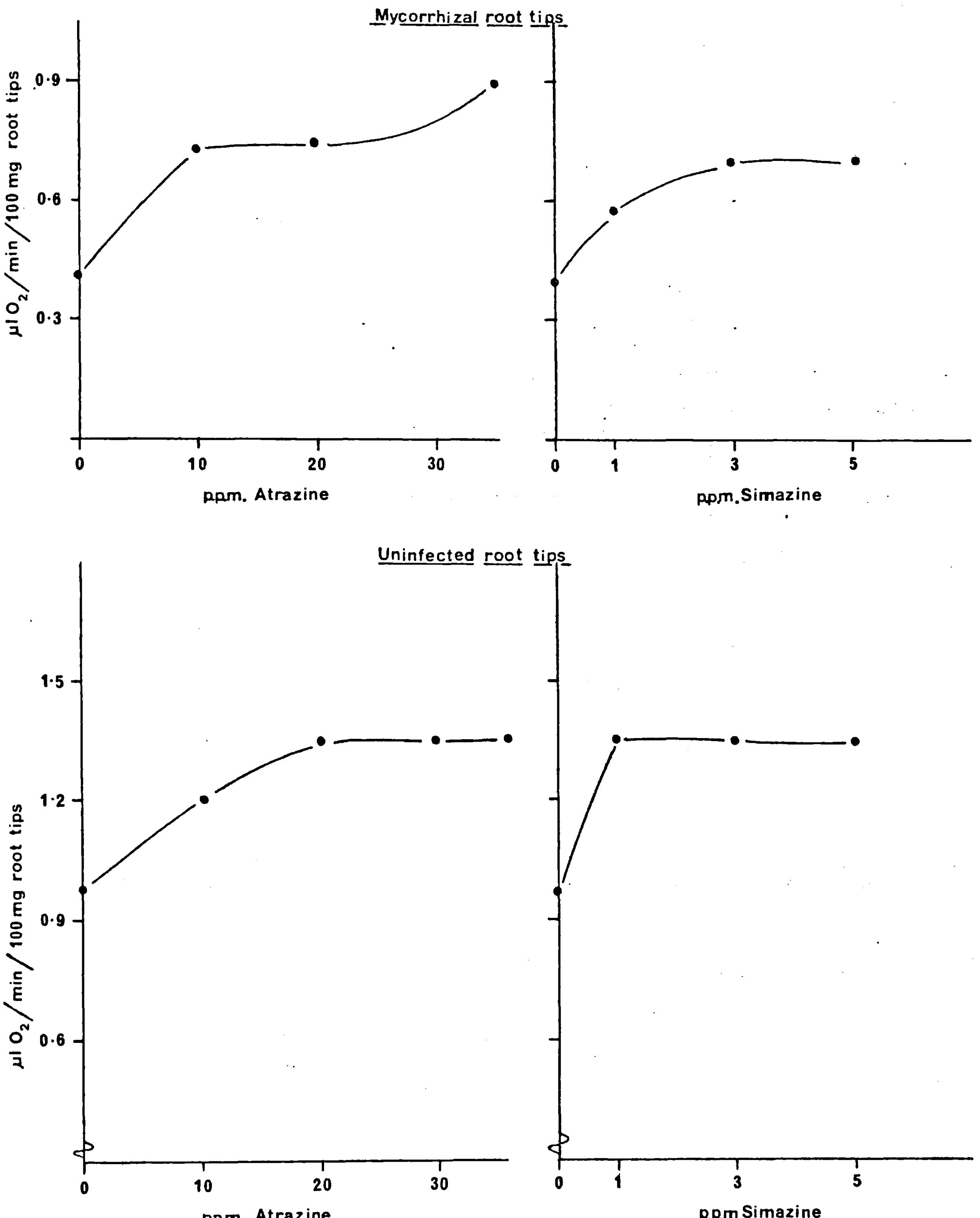
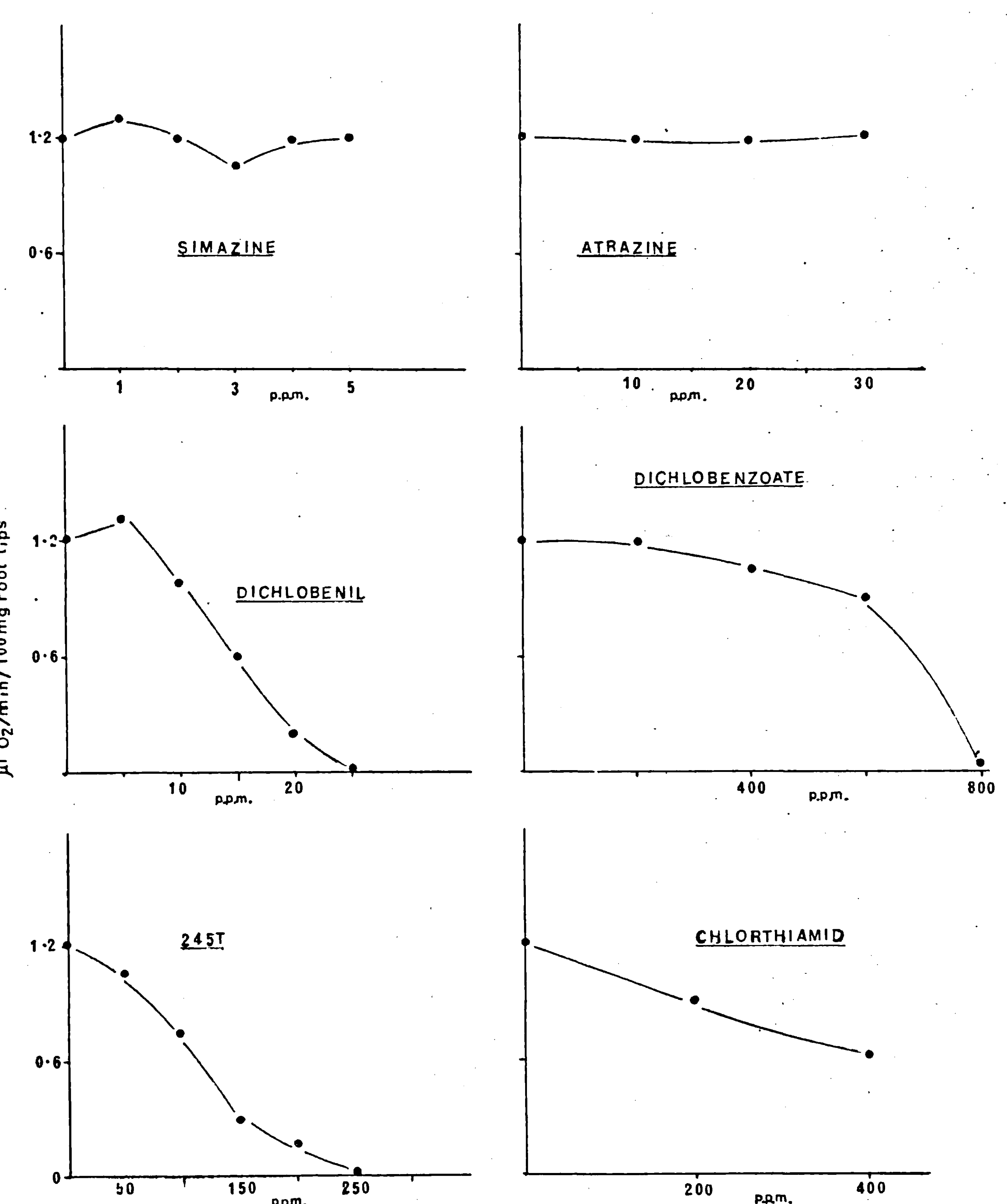


FIG. 36.

THE RESPIRATION RATES OF 100mg SAMPES OF MYCORRHIZAL ROOT TIPS OF P. SYLVESTRIS AFTER 10 MINUTES PRETREATMENT WITH VARIOUS CONCENTRATIONS OF HERBICIDES (TEMP 20°C; TREES SAMPLED SEPT. 1972).



7. THE EFFECT OF HERBICIDES ON THE RESPIRATION RATES OF MYCORRHIZAL FUNGI.

The respiration rates of B.elegans and A.rubescens, grown in shake liquid cultures, are much higher than the respiration rates of the same weight of coralloid mycorrhizas. The measured respiration rate of B.elegans is 9.25 $\mu l O_2$/minute/10 mg fresh weight of fungal pellet; a respiration rate of 4.5 $\mu l O_2$/minute/10 mg fresh weight of fungal pellet was obtained for A.rubescens. Even with temperature differences taken into account (incubation at 20°C for mycorrhizal roots and 25°C for fungal isolates), the results suggest that mycorrhizal fungal isolates, in terms of their respiratory activity, are much more active in pure culture than as developed mycorrhizas.

The effects of herbicides on the respiration rates of B.elegans and A.rubescens were estimated after ten minute pre-treatments with aerated basic liquid media containing specific concentrations of herbicide. Pretreatment with 150 ppm 245T at 25°C caused respective reductions in the respiration rates of B.elegans and A.rubescens to 77 and 91 per cent of the control (Figures 37 and 38). After ten minutes immersion at 25°C in 600 ppm Dichlobenzoate, 20 ppm Dichlobenil or 200 ppm Chlorthiamid, B.elegans showed 73, 60 and 71 per cent reductions of the respective respiration rates, relative to the control (Figure 37).

Reductions in the respiration rates of A.rubescens to 93, 84 and 89 per cent of the control are achieved by 200 ppm Dichlobenzoate,

15 ppm Dichlobenil and 400 ppm Chlorthiamid (Figure 38).

However, Simazine and Atrazine at any of the concentrations specified (Figures 37 and 38) did not affect the respiration rates of <u>B. elegans</u> and <u>A. rubescens</u>.

FIG.37.

THE RESPIRATION RATES OF 10mg SAMPLES OF BOLETUS ELEGANS AFTER 10 MINUTES PRETREATMENT WITH VARIOUS CONCENTRATIONS OF HERBICIDES (TEMP 25°C).

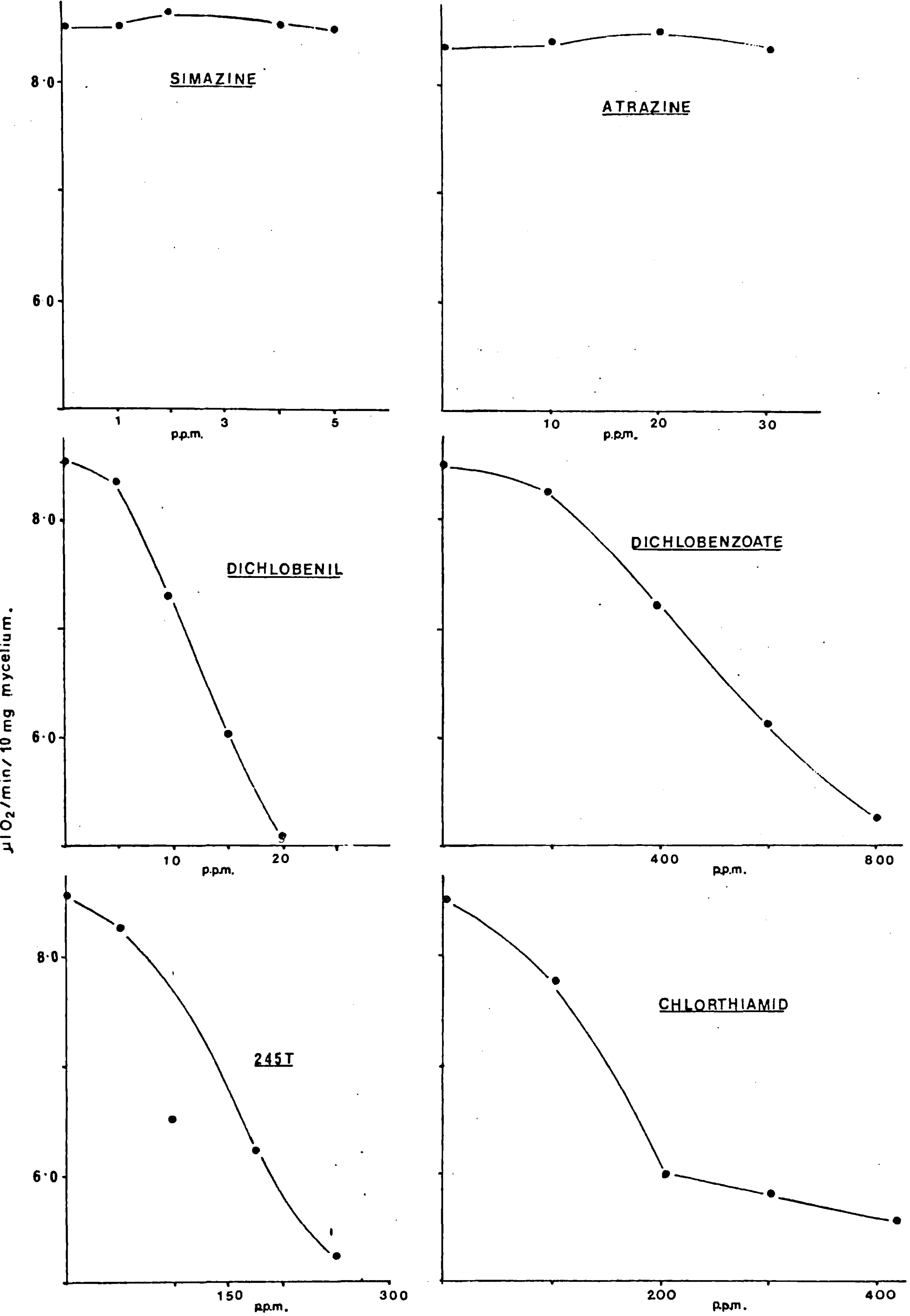
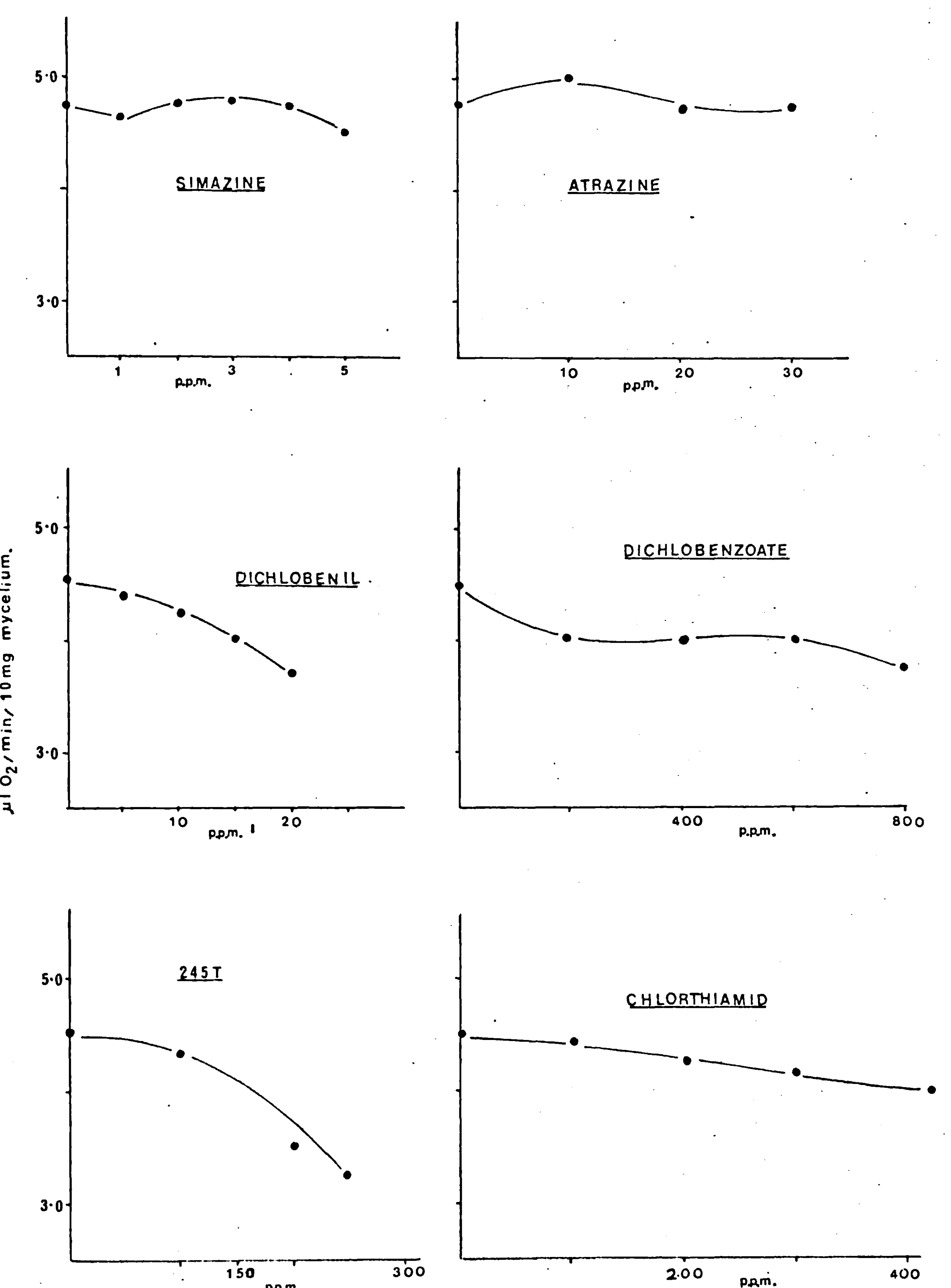


FIG.38.

THE RESPIRATION RATES OF 10mg SAMPLES OF AMANITA RUBESCENS AFTER 10 MINUTES PRETREATMENT WITH VARIOUS CONCENTRATIONS OF HERBICIDES (TEMP 25°C).



8. THE UPTAKE OF PHOSPHATE BY MYCORRHIZAL ROOTS OF P. SYLVESTRIS

Aeration of KH_2PO_4 solutions (0.05 mg/ml) in sample chambers at 20°C for 25 hours did not result in precipitation of the phosphate. Mycorrhizal roots killed with sodium azide altered the level of KH_2PO_4 in phthalate buffer, but absorption of PO_4^{3-} by these killed mycorrhizal roots was less than 0.001 mg PO_4^{3-} / 100 mg killed mycorrhizal roots / 25 hours (Figure 39 b). Any lowering of the concentration of buffered phosphate in the sample chambers containing living coralloid mycorrhizal roots (below 0.039 mg / ml PO_4^{3-}) can be attributed to the uptake of phosphate by the sample.

Aerated coralloid mycorrhizas of P. sylvestris roots began to take up PO_4^{3-} from a buffered solution containing 0.05 mg / ml KH_2PO_4 after 1.5 - 2.5 hours. After 5 - 6 hours the coralloid mycorrhizas had taken up approximately 40 per cent of the PO_4^{3-} present in the buffered solution; subsequent phosphate estimations during the remainder of the experimental period (18 - 19 hours) showed that phosphate absorption by such coralloid mycorrhizas did not increase appreciably after this time. Phosphate uptake for 100 mg coralloid mycorrhizal samples varied slightly, but in all cases absorption of PO_4^{3-} after 20 hours was between 0.14 and 0.17 mg PO_4^{3-} / 100 mg coralloid mycorrhizas (Figures 39 c and 40).

Absorption by uninfected root tips of P. sylvestris was (after 5.5 hours) in the region of 0.02 mg PO_4^{3-} / 100 mg non mycorrhizal short roots. However, after 20 hours the level of

PO_4^{3-} in the non-mycorrhizal short roots can decrease (Figure 39 a).

The uptake of PO_4^{3-} by coralloid mycorrhizas was found to be almost completely inhibited by the addition of 1 ppm 245T to the sample chamber (Figure 39). The addition to the sample chambers of 120 ppm Chlorthiamid, 60 ppm Dichlobenzoate or 25 ppm Dichlobenzonitrile caused approximately 8 - 10 per cent reductions (relative to the control) in the uptake of phosphate (Figure 40).

When reducing agent was added to buffered phosphate solutions containing Atrazine or Simazine the sample became turbid. This obscured the development of the phospho ammonium molybdate complex; hence effects of Simazine and Atrazine on the uptake of phosphate by coralloid mycorrhizas could not be measured using this technique.

FIG. 39.

THE CONCENTRATION OF KH_2PO_4 IN 10mls 0·01M PHTHALATE BUFFER (pH5·4) CONTAINING EITHER 100mg MYCORRHIZAL ROOT TIPS, OR 100mg UNINFECTED ROOT TIPS OF P. SYLVESTRIS (TEMP. = 20° C).

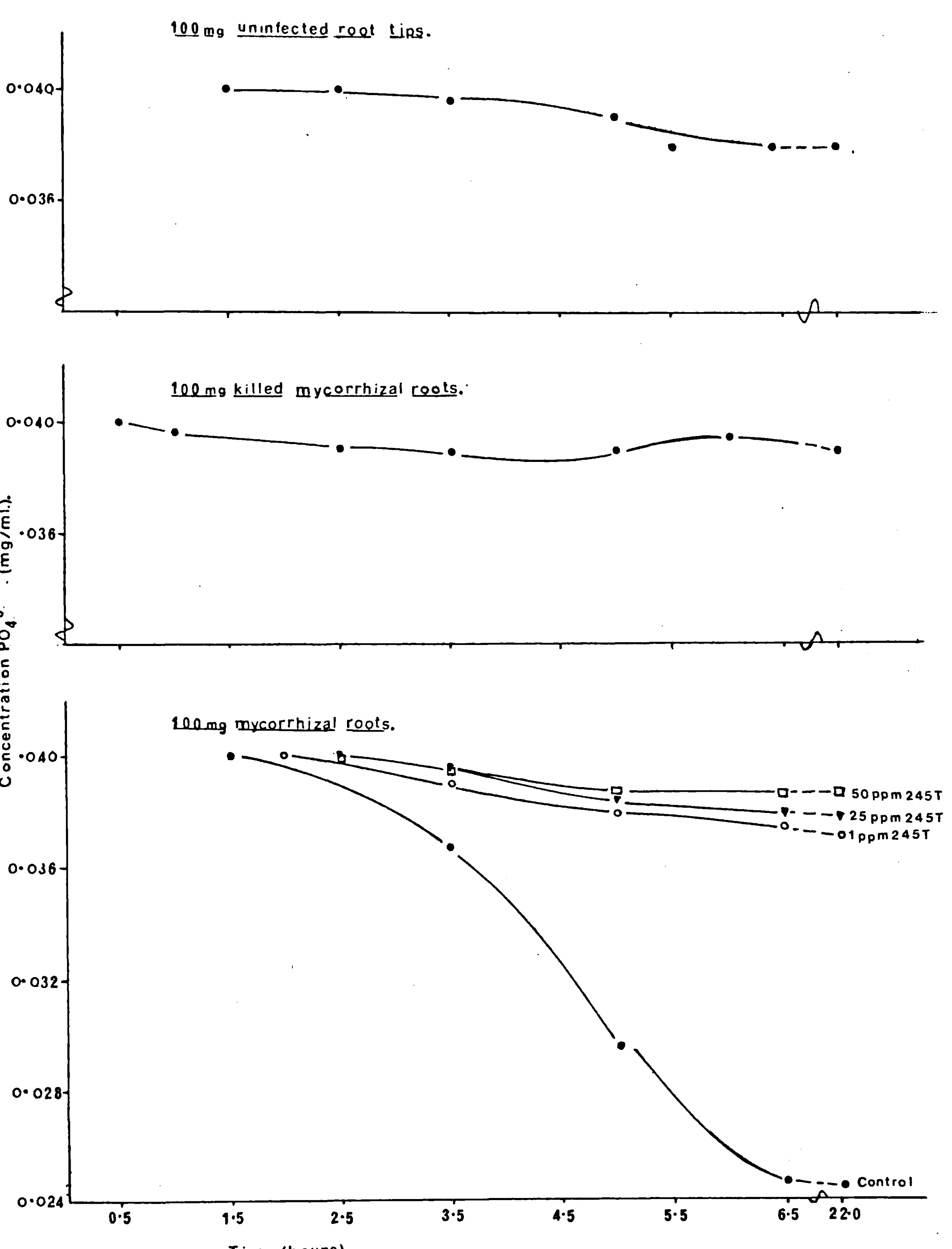
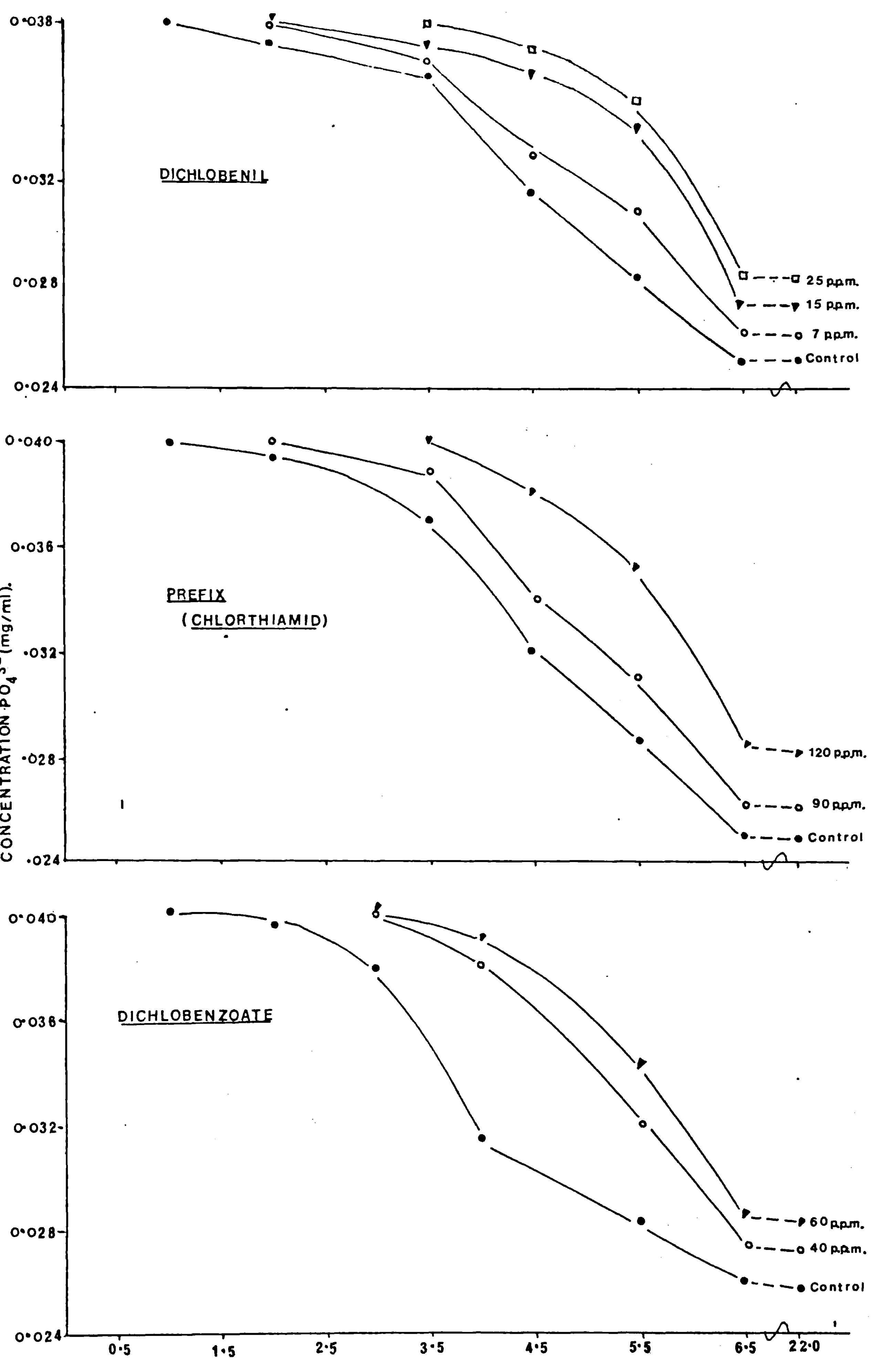


FIG. 40.

THE CONCENTRATION OF KH_2PO_4 IN 10 mls. PHTHALATE BUFFER (pH 5·4) CONTAINING 100 mg MYCORRHIZAL ROOT TIPS AND HERBICIDES AT VARIOUS CONCENTRATIONS.



DISCUSSION

1. The form and development of Pine roots.

The two Pine species examined contrasted markedly in the nature of their heterorhizic root systems. The root systems of P.nigra seedlings consisted of abundant 'pioneer' roots; the few short roots present were associated with small numbers of 'mother' root and subordinate 'mother' roots. P.sylvestris seedlings, on the other hand, possessed relatively few 'pioneer' roots while numerous short roots formed on the relatively abundant 'mother' and subordinate 'mother' roots. These differences between proportions of 'pioneer' and 'mother', and subordinate 'mother' roots on P.sylvestris and P.nigra seedlings accounted for the large differences noted in the total numbers of uninfected short root tips and mycorrhizas.

Field observations clearly show that there can be discrepancies between the establishment of mycorrhizas on Pines planted in nursery plots and on Pines naturally established in forest plantations. For example, a discrepancy was noted on comparison of nursery planted Pines with Pines planted in Thetford forest. Such discrepancies might be attributable to soil nutrient levels or to numbers of mycorrhizal propagules and their inoculum potential in forest soils compared with nursery soils. There is also the possibility that soil applied herbicides may affect either the vigour of mycorrhizal fungi or the growth and development of tree root systems. A further factor which might influence mycorrhizal development in Forestry Commission nursery plots is the use of B.H.C. for root weevil control.

High levels of soil nutrients in forest nurseries may discourage good development of root systems. For example, Söding (1952), Aird (1962), Henderson and Stone (1967) and Sutton (1969) have shown that there is a relationship between poorly developed root systems and fertile soils. In addition, a similar relationship between soil fertility and the proportions of 'pioneer', subordinate 'mother', and 'mother' roots probably exists. Thus, the overall development of the root system of a conifer seedling will depend on the particular soil nutrient levels in which it is growing (Aldrich Blake, 1930; Wilcox, 1964; Sutton, 1969).

In the present study nutrients were added at the same levels in all the experimental nursery plots; therefore overall assessments of trees within groups of herbicide treatments could be made. When Simazine is applied to soils, or soils are hand weeded, competition for nutrients between weeds (including grasses) and pine roots will be reduced. This factor may be relevant to P.nigra seedlings, for these seedlings possessed a higher 'pioneer' root: subordinate 'mother' root and 'mother' root ratio than P.sylvestris in all the nursery sites assessed. Because of this the relationship between the different site conditions and the 'pioneer' root: subordinate 'mother' root and 'mother' root ratios of P.sylvestris and P.nigra seedlings was of particular interest.

These observations concerning the effect of soil nutrients on root systems suggest that P.nigra and P.sylvestris may differ significantly regarding their ability to grow in nursery and in natural forest soils. Pines with a basic 'pioneer' root system

with few short roots can take advantage of high soil nutrient levels; however these roots may be capable of developing into secondary 'mother' root systems (Sutton, 1969). A 'pioneer' root system can be mycorrhizal in any case (Aldrich Blake, 1930; Hatch, 1937), and may finally give rise to a well developed mycorrhizal system.

Problems regarding numbers of mycorrhizal propagules and their inoculum potential in nursery and forest soils are not easily open to investigation. It seems likely that certain propagules, for example spores, would not vary appreciably in numbers and possibly inoculum potential, but others, for example rhizomorphs, might show considerable variation.

The poor development of mycorrhizas noted in forest nursery plots may be partly a consequence of the application of B.H.C. (Strobol) by the Forestry Commission to control root weevils (Table 3). Although application was only 0.5 ml B.H.C.(Strobol) / litre, this may have been sufficient to affect growth and development of new short roots. Under certain conditions B.H.C. is known to exert harmful effects on plant growth and development; for example Wegorek (1957) found that low applications of B.H.C. were harmful to germinating wheat shoots growing in sandy soil; while the same dosage of B.H.C. did not affect wheat germinating in heavy soils. However, the soil micro-flora in either B.H.C. treated soil was not affected in any distinctive way.

In the present study B.H.C. was sprayed by the Forestry Commission directly onto the roots of <u>P. sylvestris</u> and <u>P. nigra</u>.

If B.H.C. influences the resumption of short root growth, sparse mycorrhizal development at or near the ends of 'mother' roots may be a direct consequence of new short root development only taking place outside the B.H.C. treated zone.

It is of interest that Pines not treated with B.H.C., growing as pot experiments using the soils from these forest research plots, developed abundant mycorrhizas. If such pot grown Pines had been treated with B.H.C. then it would have been possible to establish whether B.H.C. affected the synthesis of mycorrhizas and/or the development of short roots.

It must be emphasised that other factors may also affect the development of the root system, for example root wounding is known to stimulate root growth (Sutton, 1967) whilst the presence of fresh or only partly humified plant residues causes some inhibition of root growth (Kononova, 1961).

Classification of mycorrhizas was restricted to a simple description of their essential characteristics, namely:

1. Non dichotomous racemose mycorrhizas (Figure 6).
2. Simply-branched mycorrhizas (Figure 15).
3. Dichotomous mycorrhizas (Figure 14).
4. Coralloid mycorrhizas (Figure 7).
5. Small white spherical structures found occasionally on dichotomous mycorrhizas (Figure 12).

The classification schemes of Melin (1927), Dominik (1959) and Boullard and Dominik (1960) were found difficult to use and interpret. Attempts to describe the colour of mycorrhizas were abandoned, for their colours changed on exposure. It was sometimes difficult to define distinct mycorrhizal morphologies since the types of mycorrhiza encountered in the assessments inter-graded. Marks (1965) suggests that this inter-gradation of morphological mycorrhizal types is probably due to several mycorrhizal fungi co-habiting the root and giving rise to different mycorrhizal morphologies in different soil conditions. Similarly, Zak and Marx (1964) recognised the existence of multiple fungus mycorrhizas which consisted of associations of Cenococcum graniforme and a quiescent fungus, the multiple fungus mycorrhiza changing in fungal composition with different soil conditions.

Equally, it is possible that one fungus might give rise to different mycorrhizal root morphologies under different soil or root conditions. However Marks (1965) suggests that different mycorrhizal types are reasonably constant, but chance invasion of a Pine short root into an area dominated by a particular soil mycorrhizal fungus population can modify a particular mycorrhizal association.

The different mycorrhizal types described in the present investigation may be a consequence of interactions between many different factors.

However, the basic problems of mycorrhizal assessment are not classificatory ones but concern assessment of seasonal growth. The

development, of mycorrhizas on P. sylvestris and P. nigra short roots was seasonal and took place in spring and late summer. Consequently any Pine roots sampled in late summer possessed both recent and older mycorrhizas. The older mycorrhizas were believed to represent both the previous spring and the previous summer's growth.

The observation of seasonal mycorrhizal development was confirmed with a sample of seedlings of P. nigra taken in February 1972 from forest plot 83FY70 (Wareham). This February assessment showed that the number of young mycorrhizas, compared with a November assessment, was very few, and that older mycorrhizas were proportionally more abundant (Table 10). Consequently it was considered that the November sampling time probably gave the better estimate of young (and presumably more active) mycorrhizas.

When nursery seedlings are transplanted root growth should not be interrupted, providing fresh root tips are present, there is sufficient soil moisture, and the soil temperature is suitable. However the possibility of a 'check' in growth on transplantation obviously exists. To allow for possible delays in the resumption of root growth, Pine seedlings were assessed in November, eight months after transplantation in March. Assuming that new short roots had developed by early summer, then November estimates of mycorrhizal intensity must include any mycorrhizas developed on these new short roots in the late summer period of growth.

The assessment of mycorrhizas in the present study includes mycorrhizas developed from an existing Hartig net and / or from soil

fungal propagules. Mycorrhizas developed from successive short roots are considered a consequence of infection by an existing Hartig net. On the other hand, coralloid mycorrhizas are believed to represent the infection of new short roots by soil fungal propagules, as probably are sparse, singly dichotomous mycorrhizal roots encountered at or near the ends of subordinate 'mother' roots, 'mother' roots or occasionally 'pioneer' roots.

2. The effect of Simazine on the growth and mycorrhizal development of P. sylvestris and P. nigra seedlings.

The technique of planting non-mycorrhizal seedlings into soil pretreated with Simazine, further annual treatment of the soil with Simazine, and assessment of consequent mycorrhizal infection of the root system, can be considered an assessment of the inoculum potential of the mycorrhizal propagules in the Simazine treated soils (Wilde, 1954). Nursery seedlings were more or less free of mycorrhizas when transplanted into Simazine treated and control soils. However, as stated previously, the plots made available for the present study received high concentrations of fertilizer and well rotted organic material (Table 3); this probably resulted in soil conditions that enabled both P. sylvestris and P. nigra to grow without the extensive development of a root system and therefore without short roots suitable for mycorrhizal development. Hence the absence of abundant mycorrhizas on assessed root systems does not imply that the soils treated with Simazine lacked mycorrhizal propagules.

It is not clear whether Simazine affects the growth and development of short roots and mycorrhizas of Pine seedlings growing on forest plots 458FY70 (Kennington) and 83FY70 (Wareham). On the whole the results suggest that there is in fact little Simazine effect on the uninfected root tips of P. sylvestris and P. nigra. Although there was an increase in the number of mycorrhizas on P. nigra seedlings sampled in November 1970 growing in Wareham (83FY70) soils treated with 8 lbs Simazine per acre, this was possibly significant but did not occur in the February 1972 sample. Similarly P. sylvestris uninfected root tips and mycorrhizas, sampled from the Kennington

site (458FY70) in November 1970, responded by some increase in numbers to the application of 4 lbs Simazine per acre; this result is possibly more valid, for additionally there was an increase in the root fresh weights of these treated Pines (Table 11). However this assessment was not repeated due to other unresolved problems of this site. However it is conceivable that Simazine does affect the Pines growing on the forest nursery plots, but that the effects are small and perhaps easily masked by other site factors.

One noticeable example of the effect of site on Pine seedling growth and development occurred at Kennington. Comparison of the ratios of root dry weight : fresh weight for P.nigra seedlings subjected to the various Simazine treatments showed that noticeable differences between plots were not related to Simazine treatments. It was observed that plots with a higher root dry weight : fresh weight ratio were localised in one corner of the experimental area; the whole site sloped towards this corner. Statistical analysis, comparing certain variates of Pines growing on this corner with those growing on the rest of the site, showed that root dry weights varied significantly, but that other measured variates were not affected. Reasons for this change in the root dry weight are unclear, but in view of this additional factor requiring consideration, it was decided not to reassess the Kennington site.

In an attempt to overcome the problem of variability of root systems noted in forest nursery plots, Pines were grown in plant pots containing soils taken from the same nursery plots (Kennington and Wareham) and from Thetford forest. These pot grown

Pines, when subsequently assessed, showed a similar inherent variability of their root systems. Overall correlations between assessed root characters were similar to those obtained from seedlings growing in nursery plots (Tables 1 and 2).

Root assessments and root counts made eight months after pot planting showed that little general extension growth of the root systems had occurred although mycorrhizal development appeared to be proceeding on a greater scale than in forest nursery plots. This may have been due to the rather different growth conditions; for example, no added fertilizer and no mulching.

It was considered that sampling these Pines eighteen months after pot planting gave a more realistic assessment of their overall growth and root development. Pines growing in pots for eighteen months developed many more root tips per plant than Pines growing in forest nursery plots for eight months. It proved too time consuming, for such pot grown Pines, to count the short root tips on a per plant basis; instead results were expressed as the numbers of root tips per 1.0 g fresh weight 'mother' root. These results were compared with similar data obtained from forest nursery plots, except that calculations for the assessment of the latter Pines were based on whole root systems and not just 'mother' roots. The values for the forest nursery plots are presumably underestimated (see Tables 10 - 14); nevertheless the intensity of short roots seems much greater on pot grown Pines. Additionally, pot grown Pines possessed greater numbers of mycorrhizas, including proportionately more coralloid types.

Differences in the nature of the root system of pot grown seedlings, compared with nursery grown seedlings, may be due to a physical effect of the plant pot which somehow alters the 'pioneer': 'mother' root ratios. For instance, the plant pot may restrict 'pioneer' root development. Such restricted 'pioneer' roots may develop into secondary 'mother' root systems. Increased development of 'mother' root systems may account for the greater numbers of short root tips developed on these pot grown seedlings; this increased development of short root tips may explain the greater numbers of mycorrhizas, including coralloid mycorrhizas, present.(Coralloid mycorrhizas were very infrequent in forest nursery plots). Increasing nutrient deficiency in pots over the duration of the experiment may have stimulated short root and consequent mycorrhizal development. In addition, denser occupation of short roots per unit volume of soil means that the short roots should encounter most of the fungal propagules present.

Analysis of pot and field experiments indicates that Simazine did exert certain effects on Pine seedlings grown under certain conditions. Simazine at 4 lbs per acre increased the numbers of mycorrhizas on Pines growing in pots for all the soil types; this effect was not properly demonstrated in the forest nursery experiments. The results obtained suggest that Simazine affects the Pine root systems in two ways; firstly by somehow increasing the numbers of Pine short root tips available for infection in particular soils and secondly by causing an increase in the mycorrhizal infection of short roots. Anyhow, it is certainly true that Simazine at any of the concentrations applied for the pot and forest nursery experiments had no detrimental effect on the ability of Pine roots to form mycorrhizas.

3. The herbicide effect on the *in vitro* growth of fungal isolates.

For measurement of growth of fungal isolates in culture it is essential to use isolates which remain constant with regard to morphological vigour. Isolates not fulfilling these requirements were rejected for the purposes of the present experiments. Such isolates might in fact have been good potential inocula for mycorrhizal syntheses, poor growth perhaps relating to a more obligate fungus / root relationship. However such isolates when tested for their ability to form mycorrhizas with aseptic Pine seedlings gave similar success rates to the more rapidly growing isolates.

The mycorrhizal fungi finally used for these experiments were derived from either sporocarps or basidiospores, the exception was *Cenococcum graniforme* (culture collection).

Fungal isolates were grown on synthetic media; this may partly account for the unsatisfactory growth of some of these isolates, since the growth of some basidiomycetes can be better on natural products media (Espenshade, 1962). However, most isolates examined grew satisfactorily on basic ion agar and for reasons of consistency it was decided to use only this agar.

Agar plates represent the most realistic and practicable means of assaying herbicide effects on mycorrhizal fungal isolates and some root pathogenic fungi. Cochrane (1958) states that most quantitative studies on the growth of fungi require a liquid medium,

he further states that the most satisfactory estimate of growth is to determine mycelial dry weight. However, Cochrane (1958) also concludes that agar plates are adequate for studies of some environmental factors, even when the results obtained do not correlate with dry weight results from liquid culture experiments.

Whether agar plates should be used for essentially chemical problems, for example the effects of herbicides, is debatable, for few experimental results exist. However, agar plates offer considerable advantages over liquid culture studies, the primary advantage being the ease of colony growth measurement.

Unlike many workers, for example Rodriguez Kabana et al (1966), the herbicides used for experiment were dissolved in water; for this reason the herbicide concentration ranges were low. Water was chosen as the herbicide solvent instead of ethanol or acetone since, firstly, the possible effects of these organic solvents on the growth of fungal isolates would otherwise arise, and secondly, water as a solvent gives herbicide concentrations that are presumably closer to those encountered in the soil. Thirdly, over a long period of incubation at 25°C, it is quite possible that some of the solvent could evaporate. Hence the solubility of the herbicide in solution could be affected, and this would change the precise concentration of herbicide in strict solution in the agar.

The results obtained were to some extent predictable, and justify claims that this assay technique is sensitive. Many of the isolates growing on agar plates containing Atrazine or Simazine showed

an initial lag of eight to ten days compared with the control; this was followed by a period of growth, in some cases a stimulation was shown relative to the control growth rate. For example Armillarea mellea and Boletus granulatus were stimulated by concentrations of 0.5 ppm Simazine. The lag induced by triazine herbicides has also been observed by Rodriguez Kabana *et al* (1966), and may indicate that an adaptive period is required by the fungus to the herbicide before it can effect appreciable growth.

The results show that Dichlobenil (2,6,Dichlorobenzonitrile) is more toxic to fungal isolate growth (1 - 3 ppm causing 50 *per cent* growth inhibition of fungal isolates), than the two other related herbicides, Dichlobenzoate (2,6,Dichlorobenzoic acid) and Prefix (2,6,Dichlorothiobenzamide). Dichlobenzoate (2,6,Dichlorobenzoic acid) is a breakdown product of Dichlobenil (2,6 Dichlorobenzonitrile); the closely related herbicide Prefix (2,6 Dichlorothiobenzamide) is converted in both plants and soils to Dichlobenil (Swanson 1969). The results show that Dichlobenil is more toxic to fungal isolate growth (1 - 3 ppm causing 50 *per cent* growth inhibition of fungal isolates), than the two other related herbicides. Dichlobenzoate (the soil degradation product of Dichlobenil and Prefix) (Swanson 1969) appears to be more toxic to fungal isolates than Prefix (10 - 45 ppm and 10 - 90 ppm causing 50 *per cent* growth inhibition of fungal isolates respectively).

The results are of particular significance, since Dichlobenil, characterised by its low solubility and high toxicity to fungal growth, could cause considerable damage to a population of soil

mycorrhizal fungi. Possibly some care should be exercised in the application of these three closely related herbicides, especially Dichlobenil. However, Dichlobenil is quickly broken down in the soil to Dichlobenzoate.

However all these herbicides, like 245T, should be applied sparingly on forest nursery plantations (245T was found to cause 50 per cent growth inhibition of fungal isolates between 15 and 28 ppm).

With shake liquid cultures several problems were encountered, one of which was the selection of fungal isolates for experiment. The cultures employed were those used for agar plate assays; several of these isolates gave inconsistent growth in shake liquid culture.

Santoro and Casida (1959) have stated that fragmented mycelia are the best inocula for the liquid culture of mycorrhizal fungi, the glass beads fragmenting and exposing more hyphae to the liquid media and thereby providing a greater potential for growth. However differences in the dry weights of replicates were found even when carefully sieved mycelial macerates were used as inocula. Consequently the inoculum for the present liquid culture experiments consisted of fungal mycelia stripped from quartered 5 mm plugs of mycelium grown on basic ion agar.

The purpose of the shake liquid culture assays, as stated, was to compare overall herbicide inhibitions with those obtained from the agar plate assays; consistent results were generally obtained. It is quite clear that there was no inhibitory effect of Simazine or

Atrazine (at the concentrations used) on the growth of fungal isolates in liquid culture, upholding the results obtained with agar plates. 245T caused inhibition of all isolates in liquid culture; Prefix, Dichlobenil and Dichlobenzoate inhibited fungal isolates to a lesser extent. However, there was one noticeable exception concerning the consistency of results between liquid culture and agar plates; Russula emetica showed no inhibition of growth when cultured in liquid media containing concentrations of up to 20 ppm Dichlobenzamide (Prefix).

It should be noted that the relative growth rates obtained for the different herbicide treatments will include any effect of herbicide on the lag phase of growth.

4. The synthesis of mycorrhizas

Washing in 100 vols hydrogen peroxide followed by incubation in the dark at 25°C was a very successful means of sterilizing *P. sylvestris* seed and resulted in at least 90 *per cent* germination (Trappe, 1961). However the same sterilization procedure on *P. nigra* seeds proved to be less satisfactory, for various reasons, and no suitable alternative method of sterilization was found. Therefore, most of the synthesis work was restricted to *P. sylvestris*.

The best form of inoculum for the *in vitro* synthesis of ectotrophic mycorrhizas was macerated fungus mycelium, consisting of large numbers of hyphal tips.

Another source of fungal inoculum was mycelium stripped from 5 mm plugs of basic ion agar. Such inocula were used extensively for Melin's technique, since it was an easy matter to bury the mycelium near the growing Pine seedlings.

Considerable evidence in the literature suggests that failure of mycorrhizal isolates to form *in vitro* mycorrhizas may be attributed to the age of the fungal isolate. It may be that isolates lose the capacity to form mycorrhizas through frequent sub-culturing and mutation. Cultures maintained on slopes under mineral oil should solve this problem.

Many of the fungal isolates isolated from sporocarp tissue may be more suited to survival as saprophytes forming pseudo-mycorrhizas

rather than ectotrophic mycorrhizas. Suggs and Grand (1972) state that an *in vitro* relationship between sporocarp and tree host does not necessarily preclude the *in vivo* formation of mycorrhizas by other fungal isolates. In addition it has not been necessarily shown that *in vivo* relationships between sporocarps associated with trees and the formation of mycorrhizas always exist. Certainly, it does not follow that sporocarps associated with trees necessarily form ectotrophic mycorrhizal associations with these trees. They may instead form pseudo-mycorrhizas, or other root surface associations, or even be part of a multiple fungus mycorrhiza. Zak and Marx (1964) recognised the existence of such a multiple fungus mycorrhiza, consisting of *Cenococcum graniforme* and a quiescent fungus (neither of which form sporophores); the ratio of these two fungi changed with different soil conditions, the stimulation of one fungus suppressing the other. These observations, together with the suggestion of Watling (1971) that sporocarp tissue may consist of more than one fungus species, and the findings of Mosse (1962) that the synthesis of endotrophic mycorrhizas required a bacterial infection as well as the mycobiont (*Endogone* sp.), may point to a possible reason for the lack of success with the *in vitro* mycorrhizal syntheses.

In the isolation of the mycorrhizal fungi for the present study, the existence of multiple fungus or fungus and bacterial associations as a pre requisite for successful mycorrhizal syntheses was not considered. Since the components of the *in vitro* mycorrhizal synthesis were aseptic Pine seedlings and pure cultures of fungal isolates, it is possible that conditions were too far removed from

the normal in vivo situation for mycorrhizal synthesis. Many of the fungal isolates tested for their ability to form in vitro mycorrhizas were capable of forming a 'mantle', but not a Hartig net on host Pine roots. It was considered that such fungal isolates formed pseudo-mycorrhizas under the experimental conditions employed. Thus a problem of the correct definition of a mycorrhizal infection arose.

Trappe's technique of mycorrhizal synthesis, analagous to that of Lündeberg (1960) in that the aerial parts of the Pine are exposed to the open air, was found more satisfactory than the latter technique. The experimental conditions of Trappe's technique enable the development of true ectotrophic mycorrhizas (instead of pseudo-mycorrhizas) between fungal inoculum and Pine root. Melin's technique, which necessitates enclosing shoots in a humid sterile atmosphere, would seem less satisfactory in terms of normal shoot, and therefore seedling, development. However, neither Trappe's nor Melin's technique produced consistently high and therefore experimentally useful numbers of well developed in vitro mycorrhizas on P. sylvestris seedlings. The numbers of mycorrhizas found developed on P. sylvestris 'explants' inoculated with Thelephora terrestris or Amanita rubescens using the Fortin technique were similar to those obtained by Fortin (1966) i.e. 32 and 38 per cent respectively.

Zak and Bryan (1963), Marx and Ross (1970) and Marx and Bryant (1969) have published results showing that successful in vitro mycorrhizal syntheses are possible between certain Pines and

Thelephora terrestris; this has been confirmed by Hacskaylo (1965, and personal communication, 1970). Hence, for the present study it was concluded that *Thelephora terrestris* was possibly one of the better mycorrhizal fungi to use for the synthesis of consistently successful *in vitro* mycorrhizas. However the results obtained do not confirm this; *Thelephora terrestris* proved no better than *Amanita rubescens* for successful *in vitro* mycorrhizal synthesis.

5. The respiration rates of mycorrhizas and mycorrhizal fungi.

Results obtained using an Oxygen electrode have helped confirm the field results concerning the effect of Simazine on mycorrhizas and have additionally provided information concerning the effects of other herbicides.

The respiration rates of P. sylvestris mycorrhizal roots sampled in September were approximately three times those of equivalent P. sylvestris mycorrhizal roots sampled in March. This confirms field observations that mycorrhizal roots in September are in an active phase of growth and development, whilst mycorrhizas sampled in March are more or less quiescent. In contrast the respiration rates of uninfected short roots sampled in March and September remained almost unchanged.

The high respiration rates recorded for these uninfected short roots may in part reflect a greater degree of wounding. Nevertheless uninfected short roots, sampled in March and September, showed an increase in their respiration rate after treatment with Simazine or Atrazine.

The overall effects of various herbicides on the respiration of P. sylvestris mycorrhizas are in agreement with the findings of herbicide effects on the growth of fungal isolates on basic ion agar. It is considered that the ten minute pretreatments with high levels of herbicide for these respiratory studies are to some extent equivalent

to the effects of lower herbicide concentrations in the agar plate assays.

Simazine and Atrazine did not measurably affect the respiration rates of mycorrhizal roots sampled in September, but caused considerable increases in the respiration rates of the apparently quiescent mycorrhizas sampled in March.

Of the series Chlorthiamid (Prefix), Dichlobenil, and Dichlobenzoate; Chlorthiamid is most frequently applied to control forest weeds. This herbicide is usually applied in granular form; the granules slowly dissolve and the active herbicide washes into the soil. Hence three phases in the soil degradation of Chlorthiamid occur, each phase possibly affects the mycorrhizal roots in different ways as follows:

1. Phase of little effect other than the control of weeds (up to 200 ppm as Chlorthiamid).
2. Possible effect on mycorrhizas (as Dichlobenil up to 5 ppm), then considerable reduction in the respiration rate of mycorrhizal roots, and inhibition of mycorrhizal fungi (over 5 ppm Dichlobenil).
3. Very little effect on mycorrhizas, the herbicides having percolated through the soil and/or having been degraded to Dichlorobenzoate.

Respiration rates of 'pellets' of mycorrhizal fungi (grown in shake liquid culture) were higher, perhaps not unexpectedly,

than the respiration rates of uninfected short roots or mycorrhizas of P.sylvestris. The respiration rates of 'pellets' of Amanita rubescens and Boletus elegans showed equivalent responses to herbicides compared with similarly treated P.sylvestris mycorrhizas. Similarities between the herbicide response curves for mycorrhizas and isolated mycorrhizal fungi suggest that mycorrhizal root responses may be those of the fungal rather than the host component. However, in the absence of data concerning respiration rates of non mycorrhizal P.sylvestris roots for the complete range of herbicides, this must remain a point for speculation.

6. The uptake of phosphate by mycorrhizal roots of *P. sylvestris*

The uptake of phosphate by mycorrhizal roots was demonstrated; however the results obtained cannot easily be compared with those of Harley and McCready (1950) and Morrisson (1961) since all the sets of results are expressed on different bases.

The results indicate that herbicides induce parallel responses in phosphate uptake to respiratory responses of mycorrhizas and mycorrhizal fungi, and to growth responses of mycorrhizal fungal isolates. However, as noted, data for phosphate uptake in the presence of Simazine or Atrazine are not available, since these herbicides caused the development of turbidity on the addition to the sample solution of H_2SO_4 and ammonium molybdate. This made the estimation of phosphate in solution unreliable. Interestingly, El Dorry, Medina and Bacila (1972) have encountered a similar turbidity problem using this method of Fiske and Subarrow (1925) to measure the effects of Phenothiazine compounds on ATPase activity.

It is difficult to draw comparisons between the various experimental results, but it seems likely that 245T exerts a considerable influence on the uptake of phosphate, the respiration rates and the growth of mycorrhizal and other fungal isolates compared with the other herbicides tested; namely Simazine, Atrazine, Dichlobenil, Dichlobenzoate and Chlorthiamid.

ACKNOWLEDGEMENTS

I am very grateful to my supervisor, Dr. B. W. Ferry, for his help and guidance, and to Professor L. J. Audus for advice on the sampling techniques.

I would like to thank the Agricultural Research Council for a research assistantship to enable me to complete this work. R. M. Brown Esq., Silviculturist (Forest Research Station, Alice Hall Lodge, Bentley, Farnham, Hants.) gave much helpful advice and made the facilities of the Forestry Commission available to me.

T. W. Chapman Esq. (Shell Research Ltd., Sittingbourne, Kent) supplied Dichlobenil, Dichlobenzoate and Chlorthiamid; Geigy Ltd. (Wythenshawe, Manchester) supplied Simazine and Atrazine.

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ERRATA.

1. Incorrect order:

Rovira, A.D. - see under Ra--.

2. Ommitted:

Romell,L.G. (1938). A trenching experiment in Spruce forest, and its bearing on problems of mycotrophy. Svensk bot. Tidskr., 32 :89.

APPENDIX 1

Full chemical names of biocides mentioned in text.

1. Soil fungicides, fumigants and sterilants.

Allyl alcohol
Benomyl
Carbon disulphide
Chlorobromo propane
Chloro picrin; trichloronitromothane
'D-D', 1,3 -dichloropropane (50 per cent) + 1,2, -dichloropropane (25 per cent).
Dexon; -dimethyl amino-benzene-diazo sodium sulphonate.
Ethylene di-bromide
Formaldehyde
Formalin
Mylone; 3,5 - dimethyl tetra hydro - 1,3,5 - thiadazine-2-thione.
P.C.N.B.; pentachloranitro-benzene
Thiram; tetramethylthiuramdisulphide
Vapam; sodium N-methylthdithio-carbonate

2. Insecticides and Nematocides

B.H.C. (Strobol) benzene hexachloride

3. Herbicides

Atrazine; 2-chlor-4-ethylamino-6-isopropyl amino-1,3,5 - triazine
Chlorthiamid (Prefix); 2,6 dichlorothiobenzamide
Dichlobenil; 2,6 dichlorobenzonitrile
Dichlobenzoate; 2,6 dichloro benzoic acid
Prefix: (see Chlorthiamid)
Simazine; 2-chloro-4,6-bis (ethylamino)-1,3,5-triazine
245T: 2,4,5 trichloro phenoxyacetic acid
Weedex '50'; active ingredient 50 per cent Simazine (see Simazine).