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Utilisation of various nitrogen sources, in particular bound soil nitrogen, by mycorrhizal fungi

Mykorrhizasvampars utnyttjande av olika kvävekällor, särskilt bundet markkväve

by

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ABSTRACT

Mycorrhiza-forming species mainly of the genera *Amanita, Boletus, Rhizopogon* and *Tricholoma* and some litter-decomposing species were employed.

For ecological characterisation of the experimental material, most of these species were tested for the ability to produce the extra-cellular enzymes cellulase, pectinase, proteinase and laccase. The mycorrhiza-formers were clearly inferior to the litter-decomposers in this respect, although some deviations were noted.

Of the synthetic nitrogen sources investigated, ammonium and asparagine were most readily utilised by the fungi, followed by glycine, acetamide, nitrate and glucosamine in that order. Unusable sources were diethylamine, proline and pyridine. Nitrite had a toxic effect throughout. Litter-decomposers tended to be non-nitrate users but could more easily utilise amide nitrogen than mycorrhiza-formers.

The result from an experimental series having raw humus as the only nitrogen source indicated that the mycorrhiza-forming fungi, like most of the litter-decomposers, could utilise to a very limited extent or not at all the nitrogen bound in the humus.

The inoculation of cultures of pine seedlings on gamma-sterilised, N^{15}-labelled raw humus with pure culture suspensions of different mycorrhiza-formers and litter-decomposers influenced to varying degrees both the supply of assimilable nitrogen and plant development.

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Chapter I. General introduction

It is well known that the humus layer in boreal forests often contains large quantities of nitrogen. In spite of this, nitrogen is the plant nutrient which is most usually the limiting factor for the growth of forest trees in this climatic zone (Tamm & Carbonnier, 1961; Brantseg, 1962), since it is for the greater part bound in complex compounds and in this form is not available to trees. It must first be broken down to simple nitrogen compounds, which is considered to take place by means of enzymes produced by various kinds of organism, from bacteria to the higher fungi. The part played in this respect by the mycorrhiza-forming fungi is uncertain. Study of this group of soil microorganisms is, however, especially interesting, since mycorrhiza-formers, in contrast to most soil microorganisms, live in symbiosis with the tree roots and thus can directly influence the nutrition and development of the trees. Most of the investigations in this field have demonstrated that conifer tree seedlings possessing mycorrhiza are larger and more healthy than those which lack mycorrhiza and that they contain notably larger amounts, not only of phosphate but also of nitrogen and potassium, whether calculated per plant or as a percentage of the dry weight (Hatch, 1937; Mitchell *et al.*, 1937; McComb, 1938; Stone, 1950; Gendina, 1960 and others).

The reasons for the advantage conferred on plants by their association with ectotrophic mycorrhiza-formers have of course been discussed and several theories exist, of which the correctness of one does not necessarily exclude another. Two of these theories appear, however, to have more proponents than the others. According to one theory the favourable effect depends mainly on the fact that plants possessing mycorrhiza are provided through the external mycelium with an increased nutrient-absorbing surface, which makes it possible for them to perform better in the competition for the available nutrients in the soil (Frank, 1885; Hatch, 1937; Björkman, 1944 and others). In support of this interpretation there is, for instance, the well-known series of laboratory experiments performed by Melin & Nilsson (1950, 1952, 1953, 1955 and 1958). These experiments indicate that plants having mycorrhiza may obtain both nitrogen and many mineral nutrient elements from a source isolated from their root systems, by the agency of the mycelium. By way of a parallel to these experiments,

Ritter (1965) demonstrated that after treatment of fruit-bodies of *Hebeloma mesophaeum* with radioactive phosphate, the element could be recovered in seedlings of *Pinus silvestris* growing in the immediate vicinity of the sporophores. He obtained, however, considerably higher activity in the mycorrhiza itself than in other parts of the plant, which indicates that the phosphate accumulates at least primarily in the hyphal mantle of the mycorrhiza (cf. Harley & McCready, 1950, 1952a and 1952b). Where pine seedlings were treated with phosphate, Ritter obtained virtually no transfer of this to the fruit-bodies. In this context Stone's (1950) findings are also of interest. He grew plants of *Pinus radiata* with Sudan grass and could demonstrate that when the pine plants were provided with mycorrhiza their phosphate content increased, while it simultaneously decreased in the grass. This indicates that plants possessing mycorrhiza have a greater chance of competing for that nutrient available to them than those which lack mycorrhiza.

Another explanation of the clear effect which symbiotic association often implies for the nutrition of forest trees may be, according to Burges (1936), that the mycorrhiza-forming fungi have the ability to utilise complex compounds in the soil which cannot be utilised by the tree. Here two investigations performed by Eglite (1956) and Eglite & Jakobsone (1958) may be cited. These showed that *Boletus* species could utilise, amongst others, nucleic acids, insoluble phosphates, mica and silicates as nitrogen, phosphorus, and potassium sources. Rosendahl (see Wilde, 1954) also came to a similar conclusion. On the basis of Rayner's field experiments and her own pot-culture experiments, Levisohn (1956) concluded that inoculation of the mycorrhizal fungi *Boletus scaber* and *Rhizopogon luteolus* had a stimulating effect on the growth of forest tree seedlings and that this stimulation was evident even before the mycorrhiza had been formed. Levisohn proposed that the positive effect which the isolates of these fungi had on plant development might have been caused by their being able to break down soil organic matter and thus to release nutrients which could subsequently be utilised by the plants.

In addition to these two theories, which directly concern the plant's nutrition, may also be advanced the view held by Melin and others, namely, that the mycorrhiza-forming fungi shield the roots of the plants from the attack of pathogenic fungi and thereby contribute indirectly to their well-being. This field was intensively studied during the 1960s, and many investigations confirm this (Zak, 1964; Marx, 1967; Sasek, 1967; Hyppel, 1968 and others).

Irrespective of the way in which the ectotrophic mycorrhiza-

formers may be useful to the higher symbiont, it appears quite clear that different species can give different answers, as regards both nutrient uptake (Bowen, 1962; Ritter & Lyr, 1965; Henderson, 1968) and general plant development (Young, 1940; Moser, 1956; Henderson, 1968).

As was mentioned earlier, growth on forest land is often limited by a shortage of nitrogen in a form available to the trees, at the same time as the soil contains a large store of organically bound nitrogen which the trees cannot directly make use of: but not so the soil micro-organisms. It seems, however, to be an open question as to whether the mycorrhiza-forming fungi can make use of this nitrogen; if so, it is important to investigate whether differences exist between species in this respect, since it should then be possible to provide, already at the nursery stage, the plants with a suitable mycorrhizal partner by employing the method introduced by Moser (1963).

With the intention of elucidating this problem, a series of experiments was performed which also included a number of litter-decomposing fungi, since it appeared to be of value to be able to compare ecologically different groups of soil fungi. With the immediate aim of forming an impression of the character of those fungi intended for use in subsequent experiments, they were studied both for their ability to produce extra-cellular enzymes (Cap. III), considered to affect the decomposition of soil organic matter, and for their ability to utilise various inorganic and organic nitrogen compounds (Cap. IV). Of the following two chapters, the first concerns the ability of the fungi to utilise raw humus as a nitrogen source (Cap. V), and the second the possibility for pine plants to utilise it in the presence of mycorrhiza-forming and litter-decomposing fungi (Cap. VI).

Chapter II. General material and methods

A. Experimental material

Most of the fungus isolates employed in this investigation were cultured from sporophores collected in the Stockholm area; Tab. 1 presents the material. Some of the species were included in all four experimental series reported in the present paper while others have been used in one or two of them only. In the table the species are arranged alphabetically and since most of them are included in the check-list of Dennis, Orton & Hora (1960), the nomenclature of this has been adopted. With the guidance of the valuable compilation by Trappe (1962) of the literature concerning ectotrophic mycorrhiza-forming fungus species, for each species in the table is shown whether it is to be considered as a true mycorrhiza-former [+] or as a presumptive mycorrhiza-former [(+)]. Only when pure culture synthesis experiments have demonstrated that a species can form mycorrhiza has it been listed as a true mycorrhiza-former. In the cases in which a species is supposed to be a mycorrhiza-former, this conclusion has often been based on the ecology and taxonomy of the fungus and should in some cases be accepted with reservation. Thus, for instance, *Tricholoma nudum* has been given as a possible mycorrhiza-former both with conifers and with broad-leaved trees, whereas pure culture synthesis experiments (Modess, 1941 and Norkrans, 1949) and enzyme studies (Norkrans, 1950) indicate that this *Tricholoma* species is purely a litter-decomposer. Furthermore, it appears improbable that either *Boletus bovinus* or *B. variegatus* could act as a symbiont with broad-leaved trees, since the fruit-bodies of both of these species—at least under Swedish conditions—are found only in the immediate vicinity of pine. On the other hand, it has recently been reported that *Amanita rubescens* has formed mycorrhiza in pure culture synthesis experiments with *Pinus silvestris* (Fortin, 1966 and Lundeberg, 1967) and *Hebeloma crustuliniforme* with *Psuedotsuga menziesii* (Trappe, 1967a). In addition, there is much to indicate that, for instance, *Boletus aeruginascens* is a true mycorrhiza-former with *Larix* spp. even though this has not yet been demonstrated by means of pure culture synthesis experiments.

Table 1. List of fungus isolates used in the present work, with an indication of their ability to form mycorrhiza (according to Trappe, 1962). For explanation of symbols, see p. 8.

Species	Collection No.	Isolation year	Mycorrhiza-former with *Pinus silvestris*	other conifers	broad-leaved trees
Agaricus campestris L. ex Fr.	C 267	1966	—	—	—
Amanita citrina (Schaeff.) S. F. Gray	C 274	1966	+	+	(+)
A. muscaria (L. ex Fr.) Pers. ex Hooker	C 4	1963	+	+	+
	C 256	1964			
A. rubescens ((Pers.) Fr.) S.F. Gray	C 6	1963	(+)	+	(+)
Boletus aeruginascens Secr.	C 264	—	(+)	(+)	—
B. badius Fr.	C 8	1964	+	+	(+)
	C 251	1965			
B. bovinus L. ex Fr.	C 15	1964	+	+	(+)
	C 16	1963			
	C 252	1966			
B. edulis Bull. ex Fr.	C 18	1966	(+)	+	+
	C 93	1967			
B. elegans Schaum. ex Fr.	C 117	1967	(+)	+	—
B. granulatus L. ex Fr.	C 19	1965	+	+	—
	C 240	1964			
B. luteus L. ex Fr.	C 81	1967	+	+	—
	C 243	1964			
B. piperatus Bull. ex Fr.	C 277	1966	—	(+)	—
B. subtomentosus L. ex Fr.	C 29	1963	(+)	+	(+)
	C 30	1963			
	C 31	1963			
	C 36	1968			
	C 118	1967			
B. variegatus Sow. ex Fr.	C 35	1966	+	+	(+)
	C 79	1967			
Cenococcum graniforme (Sow.) Fred. & Winge	D 10	1961	+	+	+
Clitocybe nebularis (Batsch ex Fr.) Kummer	C 137	1967	—	—	—
Clitopilus prunulus (Scop. ex Fr.) Kummer	C 45	1951	+	(+)	(+)
Corticium bicolor Peck.	C 269	—	(+)	(+)	—
Fomes annosus (Fr.) Cke.	Sä 1	1960	—	—	—
Hebeloma crustuliniforme (Bull. ex St. Amans) Quél.	C 87	1951	(+)	(+)	+
Hygrophoropsis aurantiaca ((von Wulfen) Fr.) Maire apud Martin-Sans	C 135	1967	—	—	—
	C 245	1964			
Lactarius deliciosus (L. ex Fr.) S. F. Gray	C 154	—	+	+	—
Paxillus involutus (Batsch ex Fr.) Fr.	C 122	1963	(+)	(+)	(+)
	C 248	1964			
Pholiota mutabilis Schaeff. ex Fr.	C 262	1966	—	—	—
Rhizopogon luteolus Fr. & Nordh.	C 282	—	+	+	—
Rh. roseolus (Corda) Hollos	C 236	—	+	+	—
Stropharia aeruginosa (Curt. ex Fr.) Quél.	C 265	1966	—	—	—
Tricholoma albobrunneum (Pers. ex Fr.) Quél.	C 278	1966	+	+	—
T. imbricatum (Fr. ex Fr.) Kummer	C 223	1960	+	+	—
T. nudum (Bull. ex Fr.) Kummer	C 225	1964	—	(+)	(+)
T. pessundatum (Fr.) Quél.	C 226	1955	+	+	—

B. Determination of pH

The pH values were measured by means of a potentiometer (Beckman Zeromatic) with a glass electrode and a calomel electrode as reference. On measurement of the pH in pure raw humus or in mixtures with raw humus, twice the volume of aq. dest. was added, and the suspension was then shaken for three hours before the determination was carried out.

C. Statistical methods

Unless otherwise stated, the results are presented as averages and the following formula was used for calculation of the standard error: $\sqrt{S(x-\bar{x})^2/n(n-1)}$. Where significance tests were performed, use was made of the t-test, the *, **, and *** significance being given for the 5, 1 and 0.1 per cent levels.

D. Other material and methods

Reference is made to other material and methods in Cap. III—VI.

Chapter III. Enzymatic activity by mycorrhiza-forming and litter-decomposing fungi

A. Introduction

The enzyme pattern exhibited by a higher fungus often gives a fairly good indication of the ecological group to which it belongs. The two groups referred to here are that comprising the ectotrophic and ectendotrophic mycorrhiza-formers and that comprising the litter-decomposers, including the wood-destroying fungi.

In 1925 Melin demonstrated that the mycorrhiza-forming fungi which he tested lacked the ability to utilise cellulose as an energy source. Both How (1940) and Mikola (1948), amongst others, obtained the same result. Also Norkrans' (1950) study of *Tricholoma* species showed a similar pattern, except for the case of *T. fumosum*—a mycorrhiza-former with *Pinus silvestris*. This fungus exhibited the ability to break down cellulose to an extent equal to that of the litter-decomposers investigated. Norkrans demonstrated, moreover, that the addition of "glucose starter" meant that some of the mycorrhiza-forming species could adapt themselves, to some extent, to utilise cellulose or closely related compounds. Norkrans states that "the difference in cellulase-forming ability between litter-decomposers and mycorrhiza-formers is probably a quantitative rather than a qualitative one". Investigations by Lyr (1963a) and Ritter (1964) have subsequently confirmed this. In their experiments, practically all the mycorrhiza-forming species exhibited some cellulase activity, which was, however, less than that of the litter-decomposers (Lyr) and markedly less than that of the wood-destroying fungi.

The pattern for pectinase production was very similar to that for cellulase and from the last two papers referred to above, it appears that wood-decomposers have a high activity. For the mycorrhiza-forming species, Lyr obtained generally no activity, whereas Ritter demonstrated slight activity. Also as regards proteinase activity, Lyr demonstrated that the relationship between the ecological groups was for the most part the same as that for cellulase activity. On the other hand, neither author could demonstrate noteworthy differences in respect of the production of xylanase or amylase and Ritter could not do so for mannase.

If the production of laccase (a polyphenoloxidase) be considered, the relationship between mycorrhiza-formers and litter-decomposers seems to be more complex. Lindeberg (1948) demonstrated that all 26 species which in earlier experiments (1944 and 1946) had shown themselves capable of breaking down lignin (and cellulose), also gave a positive reaction for laccase in the so-called Bavendamm test, while 12 of 14 species which in pure culture synthesis experiments formed ectotrophic or ectendotrophic mycorrhiza either failed to produce this enzyme or did so to a very limited extent. By contrast, both *Boletus subtomentosus* and *Lactarius deliciosus*, mycorrhiza-formers with *Pinus mugo (P. montana)* (Modess, 1941) and *P. virginiana* (Vozzo & Hacskaylo, 1962), *P. silvestris* and *Picea abies* (Melin, 1925), respectively, produced laccase to the same extent as the litter-decomposers. Lindeberg also performed a confirmatory experiment with *B. subtomentosus* by culturing it on litter, the dry matter content of which was reduced by the action of the fungus to half its original value in six months. In other words, it behaved purely as a litter-decomposer. In this context it should be mentioned that Romell (1939) found fruit-bodies of this species on trenched (root-isolated) plots in forest and described the species as a facultative mycorrhiza-former, since it could persist without contact with living tree roots. The same applied to *Paxillus involutus*.

Besides *Lactarius deliciosus*, all five of the *Lactarius* species which Lindeberg tested exhibited a high laccase activity and one of the conclusions which he drew from the experiment was that there is no sharp boundary between the two ecological groups in this respect. In passing, Lyr (1963a, p. 124) also mentioned that he had found laccase in sporophores of mycorrhiza-forming species and that it appeared to be a taxonomic characteristic for, amongst others, the genera *Lactarius* and *Russula*.

With the intention of obtaining some information about the enzymatic character of some of the fungi to be included in later experiments (described in Cap. IV, V and VI), a study was made on them in respect of their ability to produce cellulase, pectinase, proteinase and laccase, all of which are extra-cellular enzymes.

B. Material and methods

1. Culture conditions

The investigation was carried out using 23 fungus isolates, of which most belonged to the group mycorrhiza-formers. The mycelium was

cultured in 100 ml Erlenmeyer flasks containing 25 ml of nutrient solution, the composition of which was generally the same as that used by Ritter (1964) in a similar investigation. The nutrient solution in the present work thus had the following composition:

Malt extract	20 g
Bone meal	5 g
Pectin	5 g
Dry yeast	0.5 g
Cellulose	0.6 g
Tannin	0—500 mg
Aq. dest.	1000 ml

The cellulose consisted of a 1 cm tall cylinder cut from a Soxhlet thimble with an internal diameter of 1 cm. The tannin concentration was initially set at 0.05 per cent, or in other words, somewhat lower than that recommended by Lyr (1958) for the Bavendamm test. All litter-decomposers tolerated this concentration, while about half of the mycorrhiza-formers showed a clear inhibition of growth, for which reason the concentration was reduced to 0.005 per cent for seven of them. Two isolates—*Boletus subtomentosus* (C 118) and *Tricholoma albobrunneum*—were inhibited by even this lower concentration, and were therefore cultured in a nutrient solution lacking tannin.

The cellulose cylinders were sterilised in an oven at 105°C overnight, while the flasks with the nutrient solution were autoclaved for 20 minutes at 1 atm. After they had cooled, the cylinder of cellulose was placed in each, lying in the solution, after which the flasks were inoculated with ca 3×3 mm pieces of inoculum—one per flask—taken from the peripheral parts of fungus cultures grown on malt agar dishes (2.5 per cent of malt extract). The piece of inoculum was placed on top of the cellulose cylinder. This procedure ensured that the mycelium was well supplied with oxygen throughout the experiment (Fig. 1). The initial pH was 4.3, irrespective of the tannin concentration. The cultures were incubated in darkness at 25°C and two parallel series, each comprising four flasks, made up each treatment. After incubation for 10, 30 and 50 days the solutions from the four flasks in each line were combined (occasionally from three because of infection) and the volume and pH were determined; the solutions were centrifuged at 3000 r.p.m. for ten minutes before the enzyme activity was measured. The mycelium from each series was transferred to dried (60°C) and weighed filter papers (Munktell No. 3) in a Büchner-funnel, where

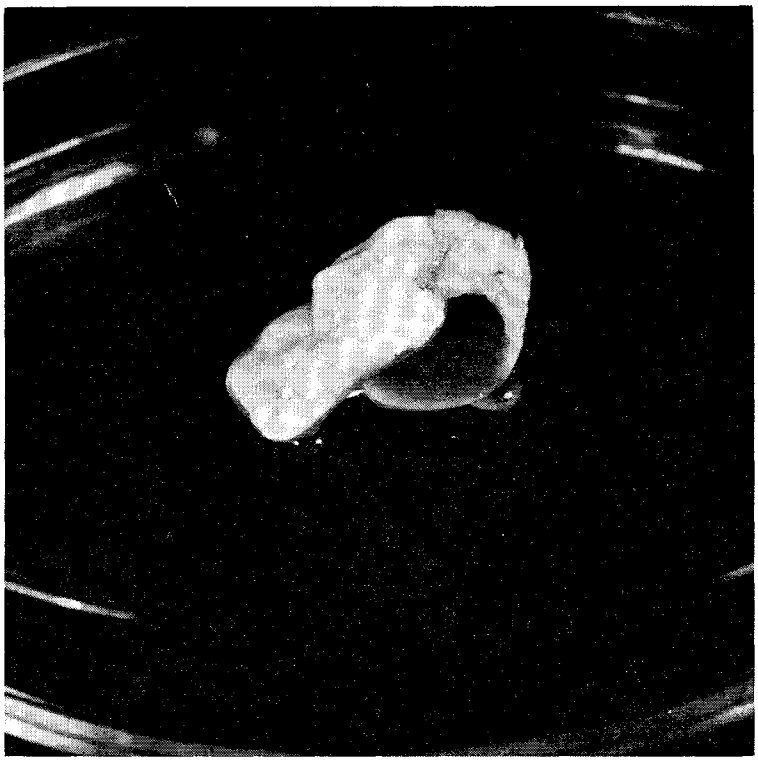


Fig. 1. Ten-day-old culture of *Amanita muscaria* inoculated on a cellulose cylinder. The culture had been removed from the Erlenmeyer flask and placed on a petri dish before being photographed.

it was thoroughly rinsed. After rinsing the mycelium was dried, well spread out, at 60°C for 24 hours, after which it was transferred to a desiccator in which it was allowed to cool before being weighed.

2. Enzyme determination

Cellulase

For this determination a Cannon-Fenske viscosimeter was used, having an efflux volume of 1.0 ml and efflux time of 6.2 seconds for distilled water at 25°C. 1.2 grammes of carboxymethyl cellulose—CMC—(manufactured by Uddeholm AB; degree of substitution 0.67; degree of polymerisation 525) was mixed for three minutes with 200 ml of 0.05 M sodium acetate buffer solution (pH 5.0) in a Turmix homogeniser of normal standard, after which the solution was placed in the cold

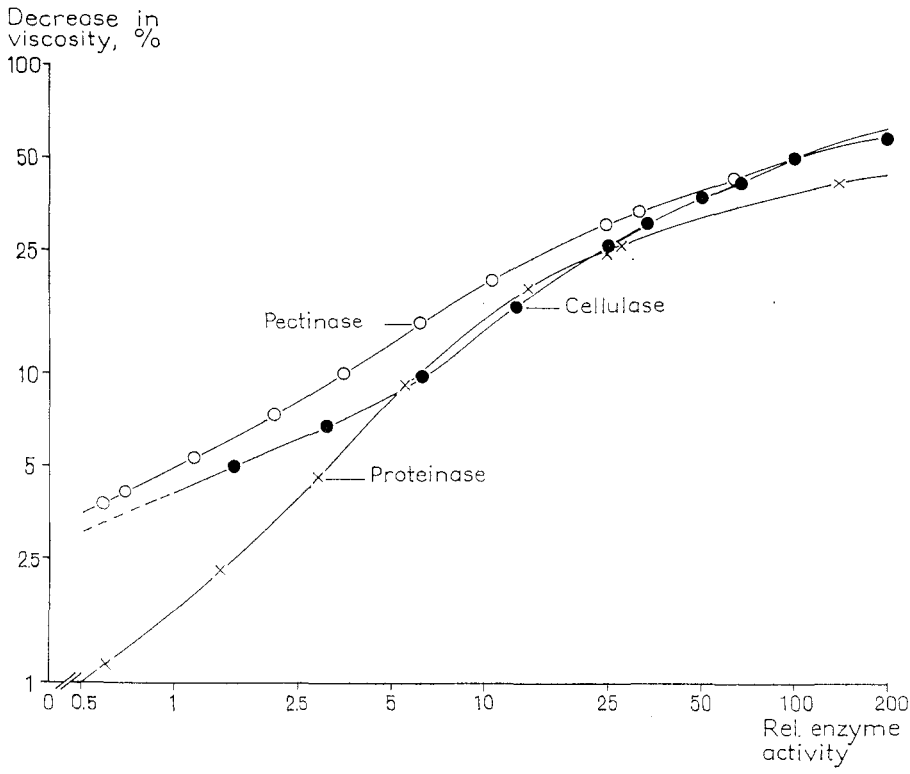


Fig. 2. Standard curve for the relationship between the decrease in viscosity and enzyme activity for cellulase, pectinase and proteinase. For further details, see text, p. 14—16.

(3°C) for 24 hours. After warming to 25°C, a vibrator was used to mix 1 ml of the fungus culture solution with 9 ml of the CMC solution. The efflux time for the mixture was determined immediately (t_0), and after 15 minutes (t_{15}), with the viscosimeter partly immersed in a water bath at 25°C. The above procedure gave t_0 values of the order of 80—100 seconds. The decrease in viscosity was given in per cent of the initial viscosity, and a solution of the enzyme the activity of which caused a reduction of 50 per cent was said to contain 100 cellulase units (after Lyr & Ziegler, 1959). A 0.004 per cent solution of a cellulase preparation from Koch-Light had such an activity. In cases in which the decrease in viscosity was greater than 50 per cent, the culture solution was diluted with distilled water and the measurement was repeated. The relative enzyme activity was obtained from the standard curve (Fig. 2).

Pectinase

In this case also a viscosimeter of the same type and capacity as that used for determining the cellulase activity was employed. 2.8 per cent of pectin (Kebo) was dissolved by heating in 0.8 per cent sodium chloride in 0.2 M sodium acetate buffer solution (pH 5.0) and was filtered hot through a glass filter funnel (Lyr & Ziegler, 1959). The decrease in viscosity was measured after 30 minutes at 30°C for a mixture of 5 ml fungal culture solution and 5 ml of pectin solution. The t_0 values were of the same order as those in the cellulase determination and in the same way the culture solution was diluted when the decrease in viscosity was greater than 50 per cent of the initial viscosity. The definition used for describing the relative pectinase activity was the same as that used for cellulase. A 0.006 per cent solution of a pectinase preparation from Koch-Light contained 100 units. The relative enzyme activity was obtained from the standard curve (Fig. 2).

Proteinase

The enzyme activity was measured with a viscosimeter of the same type as that above, but having an efflux time of 11.6 seconds for distilled water at 25°C. Six per cent of gelatine (Kebo) was dissolved in hot 0.2 M acetate buffer solution (pH 5.6). The decrease in viscosity was measured after 90 minutes at 40°C for a mixture of 1 ml culture solution and 9 ml gelatine solution (Koch & Ferrari, 1955 and Lyr & Ziegler, 1959). The t_0 values were of the order of 28—30 seconds. For a decrease in the viscosity greater than 25 per cent of the initial viscosity, the culture solution was diluted. A solution the enzyme activity of which resulted in a decrease of the initial viscosity by 25 per cent was said to contain 25 units (0.0009 per cent of a proteinase preparation by Koch-Light). The relative enzyme activity was obtained from the standard curve (Fig. 2).

Laccase

The determination was carried out at room temperature (ca 23°C) by a colorimetric procedure according to Rösch (1962). To cuvettes were added 1 ml of culture solution, 2 ml of citrate-phosphate buffer solution of pH 4.2 and 0.3 ml of guaiacol solution (1.0 g/60 ml aq. dest.). The solution was mixed with a vibrator and the absorbance was read off immediately and after 30 minutes at 480 nm on a spectro-

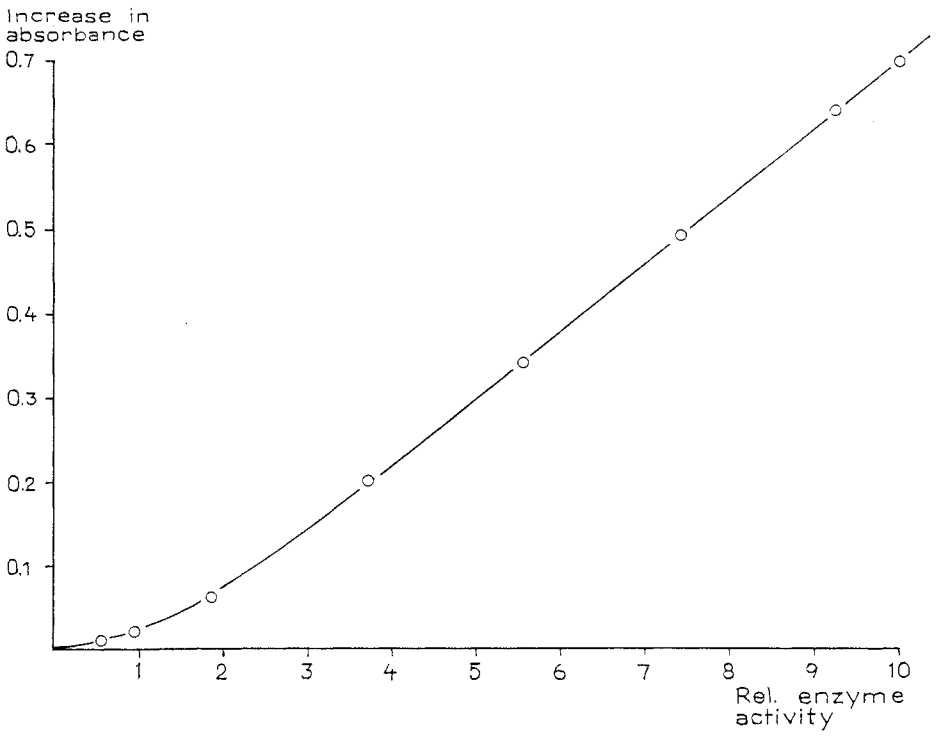


Fig. 3. Standard curve for the relationship between the increase in absorbance and enzyme activity in respect of laccase. For further details, see text, p. 16—17.

photometer (Bausch & Lomb, Spectronic 20). If the absorbance was greater than 0.7 the culture solution was diluted. The spectrophotometer was zeroed with 1 ml of aq. dest. instead of the culture solution. A solution the enzyme activity of which caused an increase of the absorbance by 0.7 was said to contain 10 laccase units. A 1.2 per cent culture solution of the composition given above (0.05 per cent tannin) had this enzyme activity 30 days after inoculation with *Agaricus campestris* (C 267). The relative enzyme activity was obtained from the standard curve (Fig. 3).

C. Results and discussion

The main aim of the investigation was to obtain partial information about the production of extra-cellular enzymes in ecologically distinct fungus species, which were subsequently to be included in experiments, rather than to make absolute comparisons between the species.

Table 2. Mycelium dry weight calculated per flask, the final pH of the solution and its relative enzyme activity in respect of cellulase, pectinase, proteinase and laccase after 10, 30 and 50 days' incubation in darkness at 25°C. The values are the average from two parallel series, each of 3—4 flasks.
The fungus isolates have been classified into 3 groups in the table. The first comprises 15 isolates of mycorrhiza-formers, the second 3 isolates of fungi of unknown ecological type and the third 5 isolates of litter-decomposers, including the root rot fungus *Fomes annosus*.

Species and Collection No.	Tannin conc. in medium ppm	Mycelium dry weight mg			Final pH			Relative enzyme activity											
								cellulase			pectinase			proteinase			laccase		
		10d	30d	50d	10d	30d	50d	10d	30d	50d	10d	30d	50d	10d	30d	50d	10d	30d	50d
Amanita muscaria C 256	50	13	32	79	4.2	4.0	3.4	<1	3	<1	0	0	0	0	<1	1	0	0	0
Amanita rubescens C 6	50	7	17	60	4.3	4.2	3.9	<1	<1	1	0	0	0	0	0	<1	0	0	0
Boletus badius C 251	50	6	14	51	4.3	4.2	4.1	<1	<1	5	0	0	0	0	0	0	0	0	0
Boletus bovinus C 15	500	28	91	153	4.3	4.3	4.3	0	0	<1	0	<1	0	<1	<1	0	0	0	0
Boletus bovinus C 16	500	29	80	176	4.3	4.3	4.0	0	0	<1	0	4	0	0	0	0	0	0	0
Boletus granulatus C 240	500	27	93	170	4.2	3.9	3.8	<1	<1	<1	0	0	0	0	0	0	0	0	0
Boletus luteus C 243	500	34	63	145	4.1	4.1	4.2	<1	<1	<1	0	0	0	0	0	<1	0	0	0
Boletus variegatus C 35	500	18	74	170	4.2	3.9	4.2	0	<1	<1	<1	<1	<1	0	0	0	0	0	0
Cenococcum graniforme D 10	50	14	63	179	4.3	4.1	3.6	<1	<1	<1	0	<1	2	0	0	0	0	0	3
Clitopilus prunulus C 45	50	13	43	173	4.3	4.2	4.1	1	171	220	0	0	0	0	4	9	0	0	0
Lactarius deliciosus C 154	50	5	17	41	4.3	4.4	4.4	0	<1	<1	0	3	<1	0	0	1	2	33	56

Table 2. ***(continued)***

Species and Collection No.	Tannin conc. in medium ppm	Mycelium dry weight mg			Final pH			Relative enzyme activity: cellulase			pectinase			proteinase			laccase		
		10d	30d	50d	10d	30d	50d	10d	30d	50d	10d	30d	50d	10d	30d	50d	10d	30d	50d
Rhizopogon luteolus C 282	500	13	72	168	4.3	4.0	3.9	2	7	<1	0	0	0	0	0	<1	0	0	0
Rhizopogon roseolus C 236	500	16	63	148	4.2	4.0	3.8	2	13	8	<1	<1	0	0	0	0	0	0	0
Tricholoma albobrunneum C 278	0	9	30	62	4.1	3.7	3.8	<1	<1	<1	5	120	235	0	0	2	0	0	0
Tricholoma pessundatum C 226	500	21	66	210	4.2	4.1	4.0	<1	<1	<1	<1	<1	<1	0	0	2	0	0	0
Boletus subtomentosus C 30	500	143	168	136	3.7	6.2	6.4	25	140	122	99	28	2	3	4	3	0	0	0
Boletus subtomentosus C 118	0	5	17	54	4.2	4.2	4.2	0	0	0	0	0	0	1	<1	<1	0	0	0
Paxillus involutus C 122	50	12	84	122	4.3	3.5	3.6	<1	<1	<1	0	0	0	0	<1	1	0	0	0
Agaricus campestris C 267	500	84	99	83	3.5	4.5	6.1	22	190	635	35	290	1300	0	2	1	500	855	905
Fomes annosus Sä 1	500	124	142	107	4.0	4.9	5.8	10	1	8	<1	5	3	<1	8	14	59	304	40
Hygrophoropsis aurantiaca C 135	500	17	149	200	3.6	3.4	3.8	10	69	30	68	283	50	2	0	0	0	0	0
Hygrophoropsis aurantiaca C 245	500	20	162	186	3.4	3.7	4.1	19	38	36	65	165	82	1	0	1	0	0	0
Tricholoma nudum C 225	500	4	20	59	4.2	4.2	3.8	<1	<1	<1	0	0	4	2	19	39	29	223	108

For this reason the culture solution in which the fungi were cultured was not diluted to its original volume, since this might have meant that in the cases in which the activity was low the lower limits for measurable activity might not have been reached. (The volume of the residual nutrient solution was on an average 20, 16 and 11 ml after 10, 30 and 50 days' incubation, respectively.) Furthermore, the varying tannin content made such a comparison impossible, as it is conceivable that the addition of this substance had an inhibiting effect on enzyme production. Mandels & Reese (1963) demonstrated that the addition of 0.33 per cent tannin caused the production of cellulase to be reduced to half in *Tricholoma viridi*, a highly sensitive organism in this respect. From the ecological point of view, the addition of tannin is fully defensible, since it and closely related compounds occur in not unimportant quantities in plants and therefore also in litter and raw humus.

Good agreement was obtained practically throughout between the two parallel series inoculated with the same isolate, but in the few cases in which there was divergence, the experiment was repeated. The results obtained are given in Tab. 2. In this the fungus species have been classed into three groups. The first comprises 14 species (15 isolates), all of which, according to the literature, have in synthesis experiments formed mycorrhiza with *Pinus* spp; an intermediate group consisting of two species (3 isolates), whose ecological classification is uncertain; and finally, the third group of four species (five isolates) of litter-decomposers, including the root rot fungus *Fomes annosus*.

Mycelium growth

For all mycorrhiza-formers and for one of the isolates of *Boletus subtomentosus* (C 118) and *Tricholoma nudum* either uniformly continuous mycelium growth was obtained during the 50 days of the experiment or usually, slightly accelerated growth between the 30th and 50th day (Fig. 4). Amongst the 15 isolates of mycorrhiza-formers could be distinguished two groups, namely, one of ten isolates with high mycelium production (145—210 mg in 50 days), and one of five isolates with low production (41—79 mg in 50 days). The litter-decomposers, including the other isolate of *B. subtomentosus* (C 30), but with the exception of *T. nudum*, exhibited another pattern, namely, either they more or less attained optimal values after ten days (*Agari-*

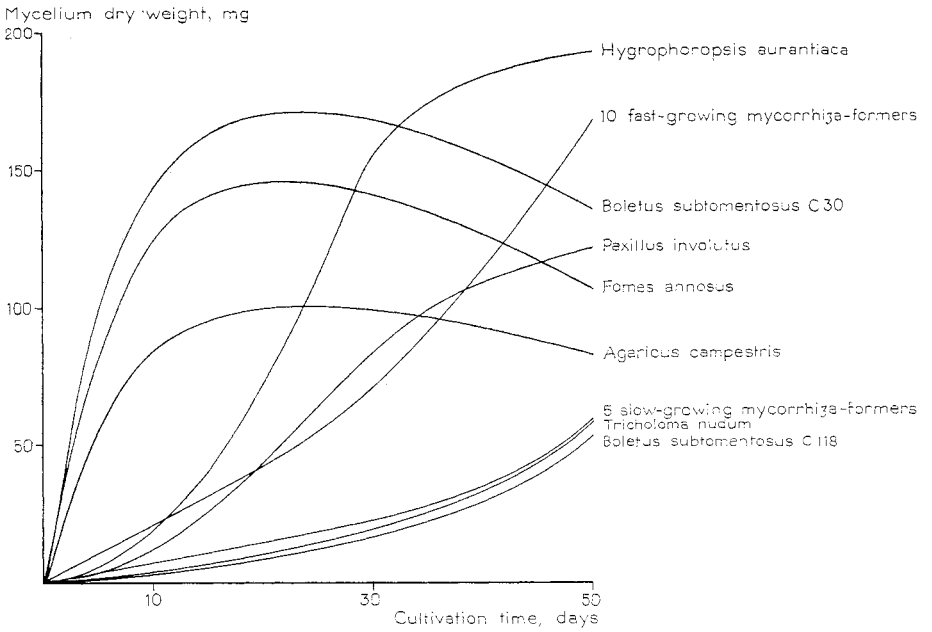


Fig. 4. Mycelium production in the nutrient solution, expressed as dry weight per flask after 10, 30 and 50 days' incubation in darkness at 25°C. The values are averages from two parallel series, each of 3—4 flasks.
For the sake of clarity the average has been given for 10 fast-growing and 5 slow-growing isolates of mycorrhiza-formers and for 2 isolates of *Hygrophoropsis aurantiaca*.

cus campestris, Fomes annosus and *B. subtomentosus* (C 30)) or had a marked increase in growth between the 10th and the 30th day (both isolates of *Hygrophoropsis aurantiaca). Paxillus involutus* exhibited a pattern which most closely resembled that of the latter. The same general difference in mycelium growth between mycorrhiza-formers and litter-decomposers was also obtained by Lyr (1963a) in his investigation.

pH

In this respect also the ecological groups were separate in that pH changes in culture solutions inoculated with mycorrhiza-formers were at a maximum of 0.8 pH units and often shifted slightly towards lower pH values. This applies also to *B. subtomentosus* (C 118) and *T. nudum*. Corresponding values for *A. campestris, F. annosus* and *B. subtomentosus* (C 30) were 2.6, 1.8 and 2.7 pH units, respectively, and after 50 days' incubation the solutions attained a pH considerably higher than

that for the other species. Cultures inoculated with *P. involutus* and both isolates of *H. aurantiaca* differed from those inoculated with mycorrhiza-formers, in that the pH values were in general somewhat lower for these three isolates.

Enzyme formation

The results from the enzyme studies generally agree well with the results of earlier investigations (Lindeberg, 1948; Lyr, 1963a and Ritter, 1964), namely, that the litter-decomposers are clearly superior to the mycorrhiza-formers as regards the production of cellulase, pectinase, proteinase and laccase. However, all mycorrhiza-formers exhibited at least at some sampling demonstrable cellulase activity, while about half of them had the ability to produce small amounts of pectinase or proteinase or both, and two of them laccase. Four of the species included amongst the mycorrhiza-formers differed, however, so notably from the others that they should be mentioned separately, namely:

(1) *Clitopilus prunulus*. The isolate of this species produced cellulase of the same order as *Agaricus campestris* which in experiments has shown itself capable of breaking down both lignin and cellulose (Waksman & McGrath, 1931); this type of litter-decomposer has been named "soil white-rots", since they decolourise humus (cf. Falck, 1930 and Romell, 1939). The group of litter-decomposers which can break down cellulose but not lignin has been named "soil brown-rots". Since the isolate of *C. prunulus* gave a negative result for laccase, but as noted above, a positive for cellulase, in this case the possibility cannot be ignored that this isolate is a soil brown-rot, even if another isolate of the same species has formed mycorrhiza (Modess, 1939, 1941).

(2) *Tricholoma albobrunneum*. This species differed from the others in the group not in respect of the production of cellulase, proteinase and laccase but rather as regards pectinase; in this context a parallel may be drawn with *Boletus elegans*, which according to How (1940), could utilise pectin as an energy source but not cellulose and lignin.

(3) *Cenococcum graniforme* and (4) *Lactarius deliciosus*. Both of these species exhibited laccase activity and thus differed from the other 12 mycorrhiza-formers. The two species also differed between themselves, in that *C. graniforme* gave a positive reaction only at the last sampling while *L. deliciosus* did so throughout.

As was mentioned by way of introduction, the litter-decomposing fungi were in general both quantitatively and qualitatively superior to the mycorrhiza-formers as regards the production of the enzymes studied. The group, however, showed itself to be highly heterogeneous and only *Agaricus campestris* and *Fomes annosus* gave a positive reaction for all four enzymes. *Hygrophoropsis aurantiaca* exhibited no activity in respect of laccase, which was in agreement with Lindeberg's (1948) result, but showed high cellulase and pectinase activity, which indicates that it might well be a soil brown-rot fungus (cf. *Clitopilus prunulus)*. Finally, the result for *Tricholoma nudum* was somewhat surprising, in that it showed the isolate's almost total inability to form cellulase. This result is completely at variance with that found by Norkrans (1950), when she demonstrated that this species could use cellulase as a carbon source even without the addition of "glucose starter". The isolate in the present work showed, however, high values for proteinase and laccase activity, which indicates that it has been placed in the correct ecological group.

Of the three species in the ecologically somewhat doubtful intermediate group, *Paxillus involutus* and *Boletus subtomentosus* (C 118) exhibited an enzyme pattern in full agreement with that for the mycorrhiza-formers, whereas *B. subtomentosus* (C 30) behaved like the investigated litter-decomposers and amongst these primarily like *Hygrophoropsis aurantiaca,* since neither of them produced laccase in the present investigation.

The interesting discrepancy existing between the two isolates of *B. subtomentosus* led to the investigation of the growth on malt agar dishes (2.5 per cent malt extract) of five isolates of this species; the laccase activity was studied by means of the Bavendamm test (0.1 per cent tannin in malt agar, Lyr, 1958) and the mycorrhiza-forming ability in pure culture synthesis experiments (Melin, 1936, p. 1082 and Modess, 1941, p. 56) with *Pinus mugo, P. silvestris* and *P. virginiana.* The result of the investigation is given in Tab. 3, in which have also been included data from the above work and works concerning experiments on this species referred to earlier in the present paper. From the composite picture hereby obtained it is apparent that *B. subtomentosus* is a variable species and that at least two different races may be distinguished, namely:

— a mycorrhiza-forming race having slow growth and low enzyme potential. Isolate C 118 certainly belongs to this, as probably do also C 31 and C 36, despite the fact that they did not form mycorrhiza in the synthesis experiments; this may have depended on the possi-

Table 3. Diameter growth on malt agar dishes, ability to form mycorrhiza with *Pinus* spp. and laccase activity in five isolates of *Boletus subtomentosus*. Data from the enzyme experiment in the present work and from other investigations of the species also included.

Isolate	Year of isolation	Diameter growth per day, mm	Mycorrhiza formation with			Laccase activity	Cellulase activity
			P. mugo	*P. silvestris*	*P. virginiana*		
C 29	1963	5	—	—	—	+ +	
C 30	1963	16	—	—	—	(+)	+ +
C 31	1963	1.0	—	—	—	—	
C 36	1968	0.5	—	—	—	—	
C 118	1967	1.5	+	+	+	—	—
Modess, 1941		1.0	+	—			
Lindeberg, 1948						+ +	+ +
Vozzo & Hacskaylo, 1962					+		

bility that the mycelium of these extremely slow-growing isolates never came into contact with roots. Modess' isolate also shows good racial affinity with the above-mentioned isolates.

— a race resembling the litter-decomposers, having rapid growth and high enzyme potential, represented by the isolates C 29 and C 30. These two differed, however, in that the latter gave only a slight indication of laccase activity, while the former showed strong activity (equivalent to *Agaricus campestris,* included in the test because of its character as a good laccase producer). C 29 behaved in this respect similarly to Lindeberg's isolate.

The conclusion to be drawn from this experiment is that if one isolate of a species can form mycorrhiza this does not necessarily apply to all isolates of the species. Each isolate should thus be considered unique, having a character which can only be discovered by investigation. How often discrepancies of the type found amongst the isolates of *B. subtomentosus* occur, for instance, as regards mycorrhiza-formation, is difficult to say, since information in the literature usually refers to cases in which mycorrhiza has been established, and rarely to negative results. Generally, however, different isolates of the same species resemble one another in a striking fashion, in colour, form and growth-rate when cultivated on malt agar. The only exceptions noted by the present author have concerned isolates of *B. edulis, B. scaber* (cf.

Levisohn, 1959) and *B. subtomentosus*, which, at least for the last-mentioned species, was also reflected in differences in enzyme production.

In summary, the results show, in conformity with those in earlier investigations, that of the two ecologically dissimilar groups of fungi tested, the mycorrhiza-forming species were clearly inferior to the litter-decomposers in the production of enzymes of the type considered to be important in connection with the break-down of organic matter in nature. The groups also differed in respect of mycelium development and pH effect on the substrate. Some deviations could, however, be noted. Thus isolates of the mycorrhiza-forming species *Clitopilus prunulus, Lactarius deliciosus* and *Tricholoma albobrunneum* produced, respectively, cellulase and proteinase, laccase and pectinase to a comparatively large extent, while an isolate of *T. nudum*, a supposed litter-decomposer, exhibited low cellulase and pectinase activity. Amongst the isolates of *Boletus subtomentosus* two distinct races could be discerned, the one unequivocally like the mycorrhiza-formers in its behaviour, the other resembling the litter-decomposers.

Chapter IV. Utilisation of various inorganic and organic compounds as a nitrogen source by mycorrhiza-forming and litter-decomposing fungi

A. Introduction

In order that the higher symbiont in an established mycorrhiza shall be able, with the aid of the lower, to obtain its nitrogen supply, it is necessary that certain conditions be fulfilled. Amongst other things, the lower symbiont, i.e. the fungus, must probably itself be capable of utilising the nitrogen source or sources available. Carrodus (1966, 1967) has demonstrated that birch mycorrhizas are scarcely capable of taking up nitrogen in the form of nitrate but can do so when it is in the form of ammonium and simple organic compounds. This inability to utilise nitrate is, in contrast to most of the lower fungi, by no means unusual in the basidiomycetes (see below), amongst which most mycorrhiza-formers are found. Furthermore, the mycorrhiza-forming fungus must at least partly be able to transfer the nitrogen taken up to its partner, and that this occurs Melin & Nilsson (1952, 1953) have demonstrated by means of their elegant method employing N^{15}-labelled ammonium nitrate ($N^{15}H_4NO_3$) and glutamic acid.

Whereas for many groups of lower fungi there has been fairly close investigation of what nitrogen sources can be accepted, knowledge of this is, however, fairly limited for both mycorrhiza-forming and litter-decomposing basidiomycetes. Most available information concerns only individual species of different genera and few genera are as well studied as *Tricholoma* (Norkrans, 1950, 1953 and Rawald, 1963). This lack of information may exist because the results of investigations have not been published (Melin, 1953), which may have been caused by their having shown disparity, without agreement either between species in the same genus or within one and the same ecological group. The nitrogen sources mainly studied have been ammonium, nitrate and some amino acids. A synthesis of the results obtained is presented below, in which special attention is paid to the mycorrhiza-forming species.

Atmospheric nitrogen

No firm evidence exists to show that mycorrhiza-forming fungi are able to fix atmospheric nitrogen, even if a few investigations (summarised by Harley, 1969, p. 134) have given positive indication of this. The possibility cannot be excluded that bacteria have been responsible for the fixation; Foster & Marks' (1967) discovery that bacteria occur abundantly in the hyphal mantle of mycorrhiza does nothing to render this supposition less likely.

Ammonium nitrogen

One of the best nitrogen sources is, without doubt, ammonium, which can be utilised by most fungi. Some, however, apparently lack the ability to synthesise organic nitrogen from inorganic as, for example, *Lactarius deliciosus*, which developed poorly on ammonium tartrate but well on a mixture of amino acids (Melin & Norkrans, 1948). This was also the case for *Coprinus lagopus* and *Pleurotus corticatus* (Leonian & Lilly, 1938)—both probably litter-decomposers. Of the ammonium salts, the tartrate and phosphate seem to be more suitable as nitrogen sources than, for instance, the chloride and sulphate, probably because a nutrient solution containing the two last named undergoes with time a more marked shift towards a lower pH value and becomes a less favourable environment for fungal growth (Norkrans, 1950; Rawald, 1963).

Nitrate nitrogen

This nitrogen source is interesting in that it is excellent for many groups of fungi, while most of the higher basidiomycetes seem unable to utilise it (Cochrane, 1958). Thus Lindeberg (1944) demonstrated that 12 out of 13 *Marasmius* species (litter-decomposers), Norkrans (1950) that eight out of nine *Tricholoma* species (both litter-decomposers and mycorrhiza-formers) and Trappe (1967b, referring to unpublished data) that six out of eight mycorrhiza-formers were non-nitrate users, and, in other words, lacked nitrate reductase. Rawald's (1963) result is somewhat in contradiction of Norkrans', since he could demonstrate that at least six of the nine *Tricholoma* species that he tested were nitrate users. Two of these species were identical with those with which Norkrans obtained negative results. Thus there appear to be differences both between species of the same genus and between

isolates of the same species. This last-mentioned circumstance may also be illustrated by reference to *Boletus variegatus,* of which the one isolate was a nitrate user (Melin, 1925) and the other a non-nitrate user (Mikola, 1965). Even for those species which can utilise nitrate this nitrogen source is, however, generally inferior to ammonium (Melin, 1925; How, 1940; Norkrans, 1950; Hacskaylo, Lilly & Barnett, 1954 and Mikola, 1965).

Amino acids

It is as easy to state the importance as a whole of the amino acids as a nitrogen source for higher fungi, as it is difficult to form a clear picture of their importance as individual amino acids. One or a few amino acids may be excellent nitrogen sources for one species and occasionally even better than ammonium, while others cannot be utilised; for another species the situation may be almost the reverse (Melin & Mikola, 1948; Melin & Norkrans, 1948; Norkrans, 1950; Rawald, 1963 and Mikola, 1965). In this context Norkrans' (1953) investigation should be mentioned; she obtained not only positive growth response to glutamic acid and aspartic acid in the presence of ammonium salts, but to the same extent also for their corresponding keto-acids. This result shows that the fungi tested (*Tricholoma* spp.) were not partially heterotrophic in respect of the formation of amino acids as such, but rather as regards the formation of carboxylic acids.

Other nitrogen sources

To the list of good nitrogen sources excepting ammonium, in some cases nitrate and some amino acids, there may also be added peptone, casein hydrolysate, propionamide and urea, as may be seen from the papers referred to above.

As the aim of the enzyme investigation was to obtain information about the fungus species to be included in subsequent experiments, it also appeared desirable to study their ability to utilise various nitrogen sources. The experiment comprised 38 isolates of 28 species on ten nitrogen sources, both inorganic and organic.

B. Material and methods

To 100 ml Erlenmeyer flasks were added 20 ml of a nutrient solution having the following composition:

Glucose	5.0 g
KH_2PO_4	1.0 g
$MgSO_4 \cdot 7\ H_2O$	0.5 g
$CaCl_2$	50 mg
$MnSO_4 \cdot 7\ H_2O$	10 mg
Ferric citrate	3.3 mg
$ZnSO_4 \cdot 7\ H_2O$	4.4 mg
Thiamine	50 μg
Folic acid	100 μg
Biotin	10 μg
Aq. dest.	1000 ml

The flasks were autoclaved at 1 atm. for 20 minutes and after cooling was added 5 ml of a sterile filtrate (Millipore GS 0.22 μ) containing the nitrogen compound to be tested, in such concentration that the final solution contained an equivalent quantity of nitrogen as a 1.0 g ammonium tartrate solution contains per litre, i.e. 152 mg. To the flasks used as controls was added 5 ml of sterile aq. dest. Before the nutrient solution was autoclaved 0.1 M sodium hydroxide or hydrochloric acid was added where necessary, in such a quantity that the final solution containing the nitrogen had a pH of 5.4.

The nitrogen sources tested were as follows:
ammonium tartrate (p.a., Kebo), potassium nitrate (p.a., Merck), potassium nitrite (p.a., Merck), acetamide (p.a., Matheson, Coleman & Bell), diethylamine (synthesis, Merck), D-glucosamine (puris, Merck), glycine (for analysis, Merck), L-asparagine (biopure, Merck), L-proline (biopure, Merck) and pyridine (p.a., Kebo).

The flasks were inoculated with material similar to that described in Cap. III (p. 13) and the pieces of inoculum were floated on to the surface of the nutrient solution. The cultures were incubated in darkness at 25°C for 21 days. The mycelium production was determined as dry weight by the procedure already described (p. 13) and the final pH of the nutrient solution was measured. In each experimental series there were four flasks; thus the whole experiment comprised ca 1650 flasks.

C. Results and discussion

To give as general a picture as possible, only the average values for the obtained mycelium dry weights for each nitrogen source are shown in Fig. 5. For information about the standard error, calculation of

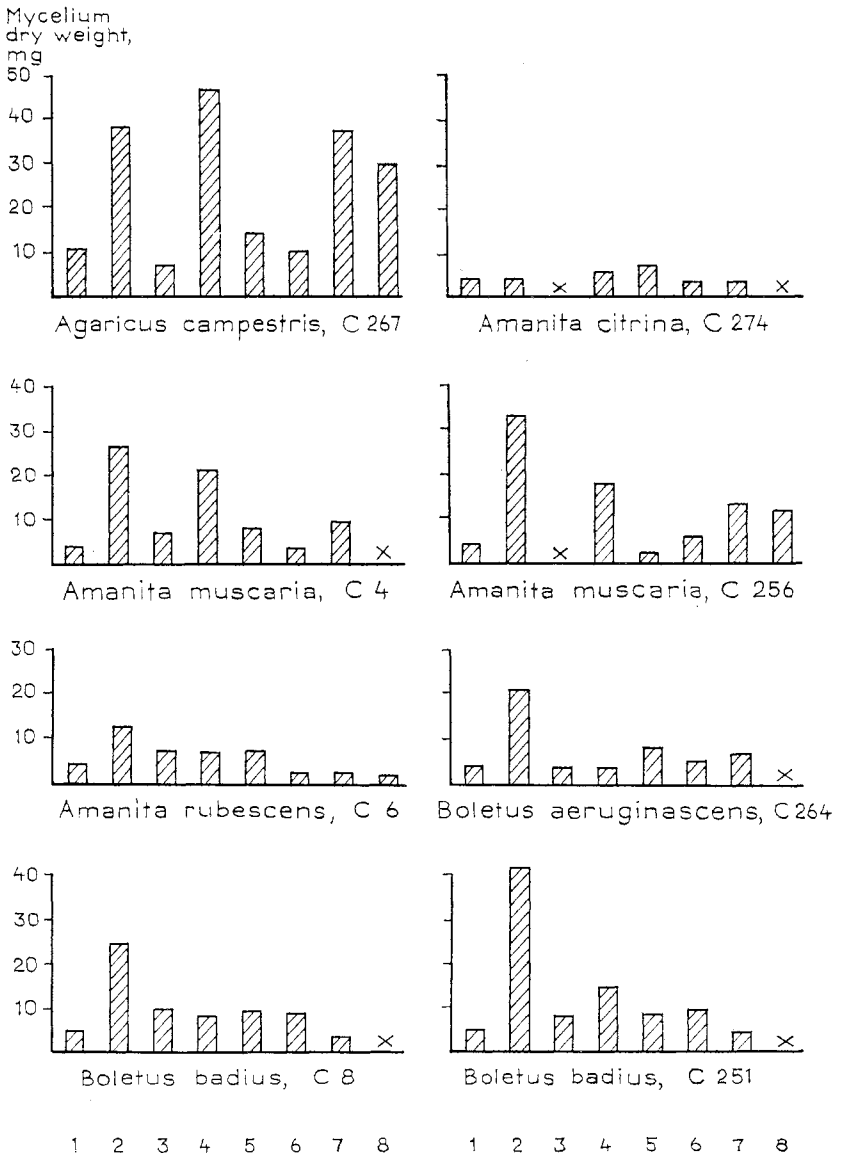


Fig. 5. Mycelium dry weight on various nitrogen sources after three weeks' incubation in darkness at 25°C. The values are the average of four determinations. The columns represent, from left to right:

1. control without nitrogen
2. ammonium tartrate
3. potassium nitrate
4. acetamide
5. diethylamine
6. D-glucosamine
7. glycine
8. L-asparagine

× in place of a column indicates that no mycelium was produced. For information about standard error, calculation of significance and final pH, see Tab. I in Appdx.

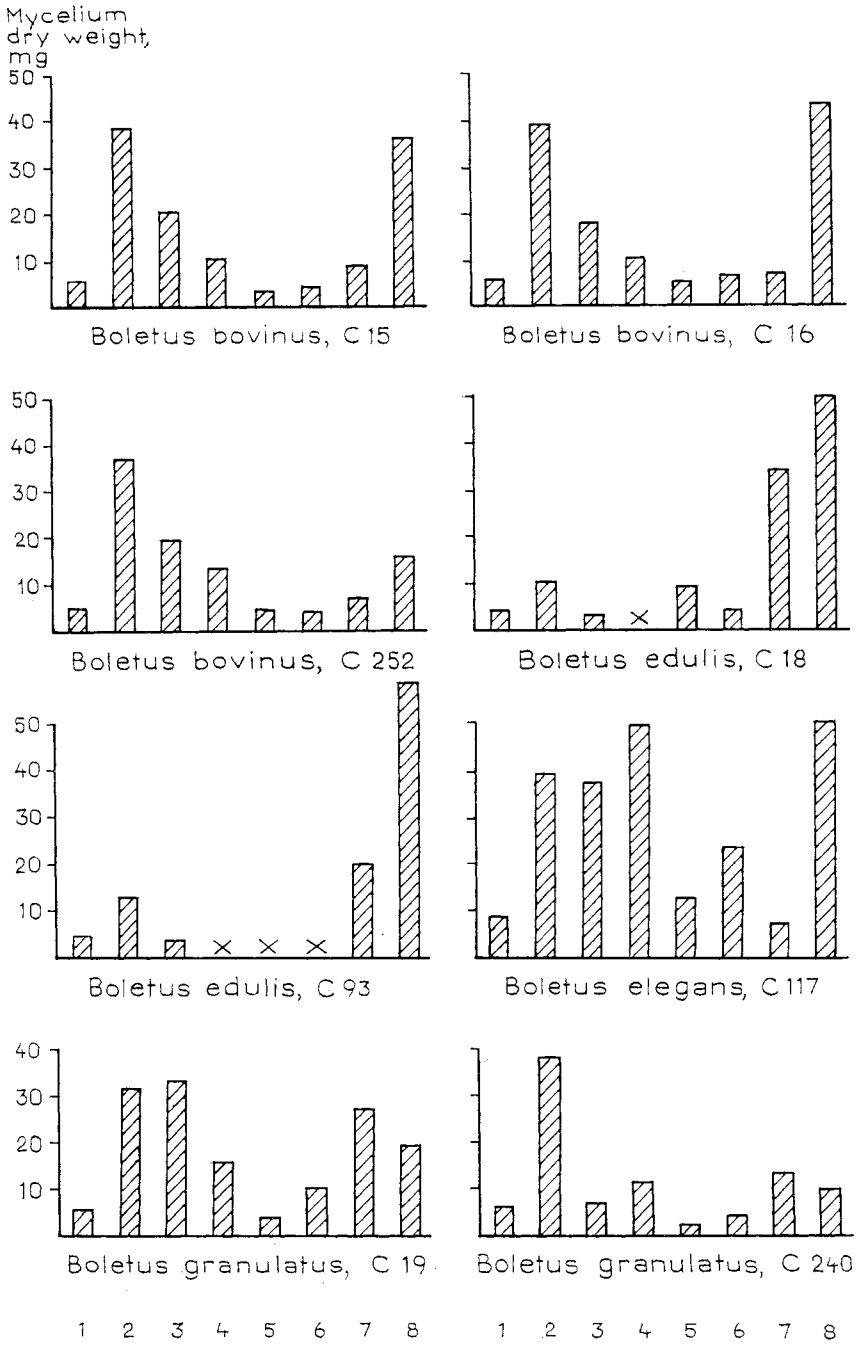
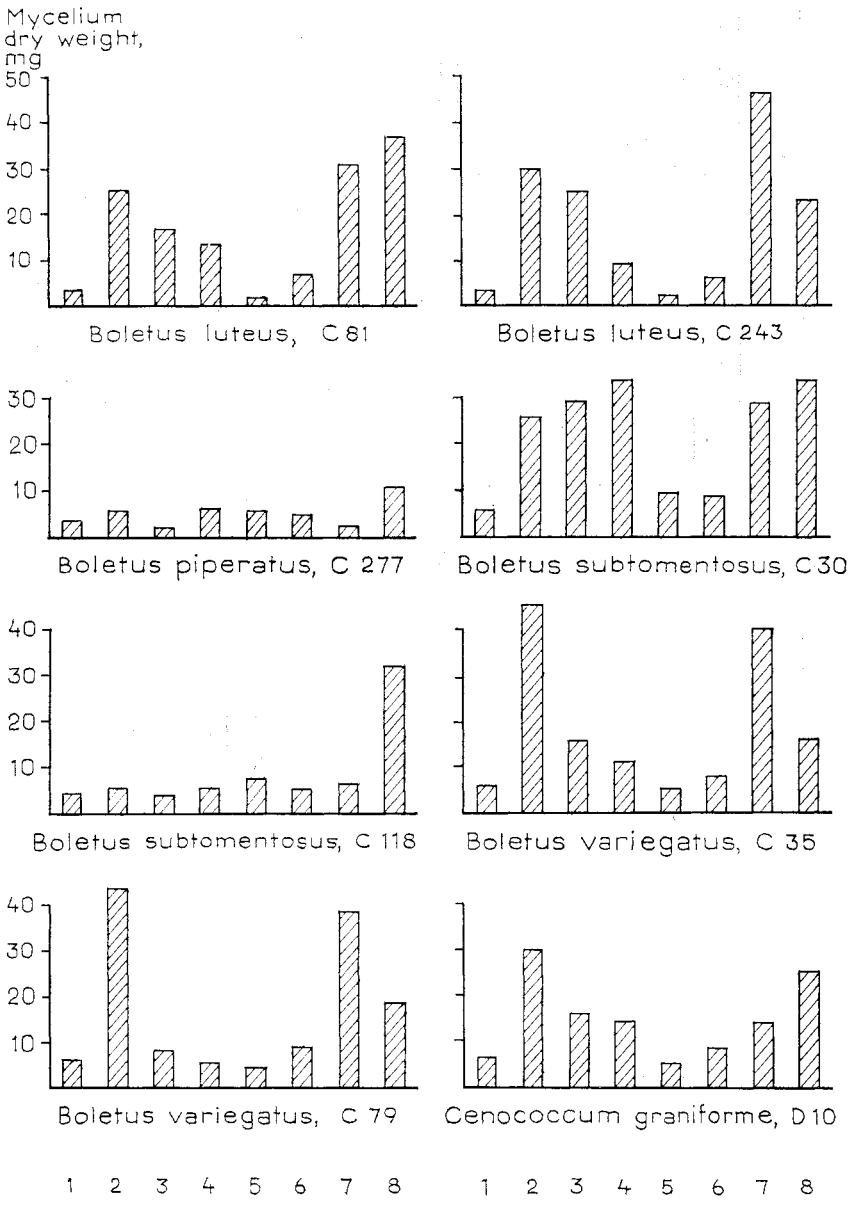


Fig. 5. *(continued)*.



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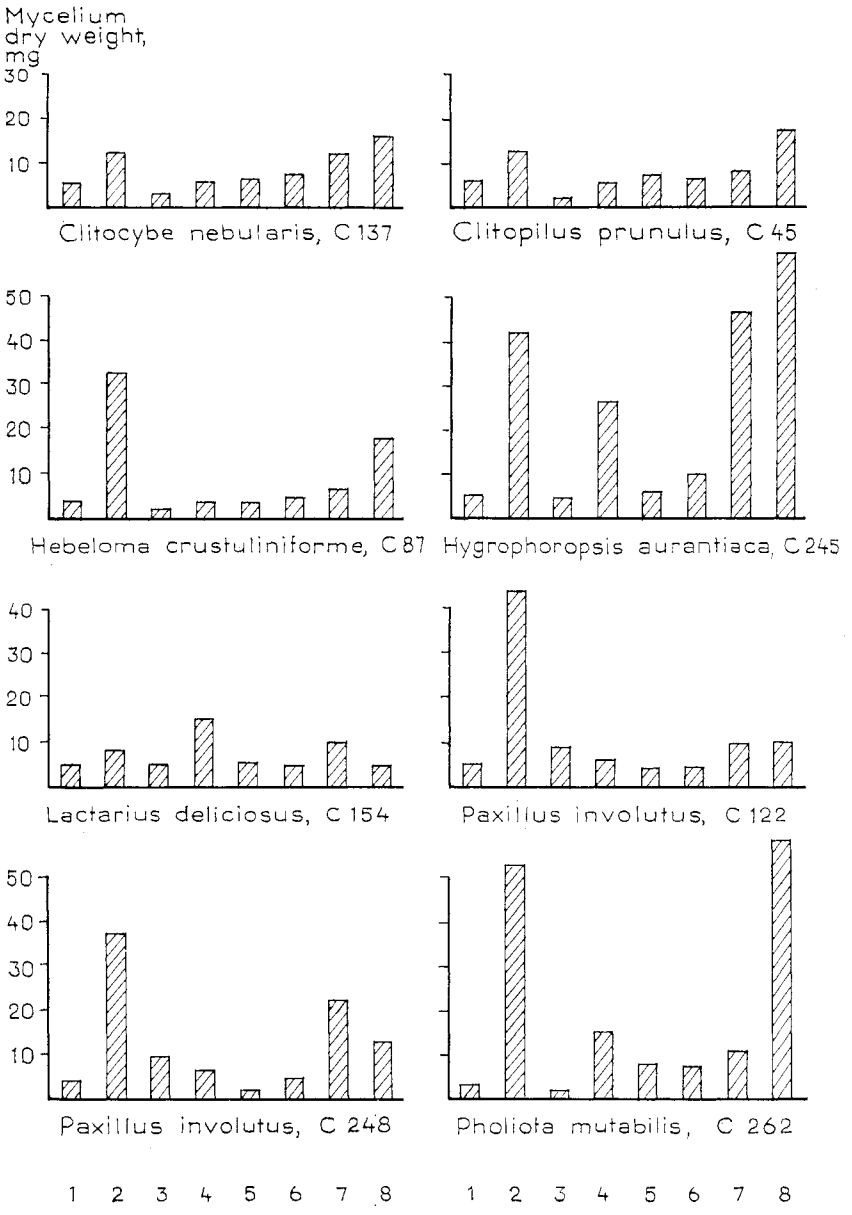

Fig. 5. *(continued)*.

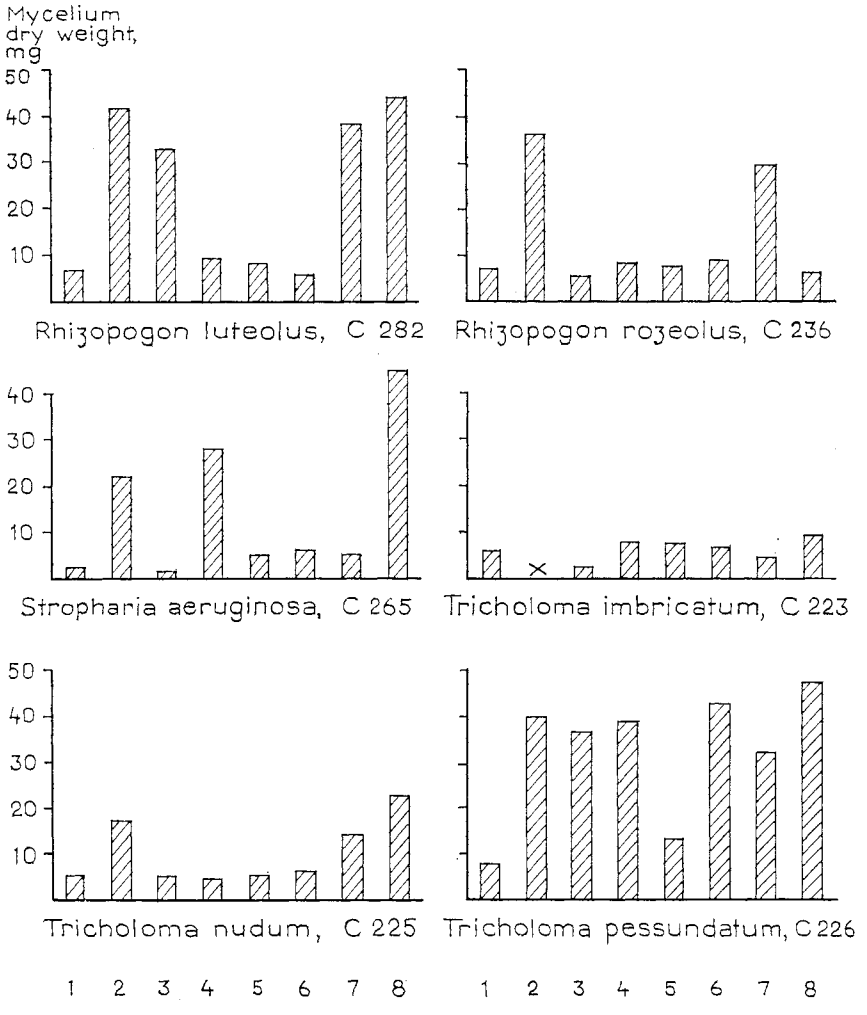

Fig. 5. *(continued).*

significance and the final pH, the reader is referred to the table in the Appendix. Here the ecological type of the fungi is also given, and on the basis of the results of the enzyme investigation (Cap. III), isolate C 30 of *Boletus subtomentosus* has been referred to the group litter-decomposers and isolate C 118 of the same species to the group mycorrhiza-formers. For the same reason *Clitopilus prunulus,* which in the previous chapter was classified as a mycorrhiza-former, was transferred from this group to that of fungi of unknown ecological type. The results from that part of the experiment, in which nitrite, proline and pyridine were used, are reported only in the Appendix, since none of these substances could be accepted as a nitrogen source by any of the fungus isolates studied.

Ammonium nitrogen

This nitrogen source, together with L-asparagine, was on the whole superior to the others tested; this result is in good agreement with the majority of earlier investigations on single nitrogen sources (Melin, 1925; Melin & Mikola, 1948; Norkrans, 1950 and Mikola, 1965). A number of clear deviations could, however, be observed. Thus five of the 38 isolates could not in general utilise ammonium, but even amongst these five there were differences in the pattern of behaviour. *Amanita citrina* and *Tricholoma imbricatum* developed poorly on all the nitrogen sources investigated, while *Boletus piperatus* and *Lactarius deliciosus* could to some extent make use of asparagine and acetamide, respectively, and possibly glycine. For the mycorrhiza-forming isolate of *B. subtomentosus,* the situation was somewhat different, since asparagine gave in this case a clearly positive growth response. Also for *B. edulis* and *Stropharia aeruginosa,* both of which could utilise ammonium to some extent, asparagine was above all a clearly better nitrogen source, and the correspondence with Melin's (1955) isolate of *B. edulis* was striking. The more or less pronounced demand shown by these fungi for organic nitrogen in order to be able to develop indicates that they are absolutely or partly heterotrophic in respect of nitrogen. Heterotrophy of this kind has previously been noted amongst mycorrhiza-formers such as *Cenococcum graniforme* (Mikola, 1948; Melin & Mikola, 1948), *Lactarius deliciosus* (Melin & Norkrans, 1948) and species of the genera *Amanita, Boletus, Cortinarius, Rhizopogon* and *Russula* (Melin, 1953, 1955).

Nitrate nitrogen

In general, and in good agreement with earlier investigations (see Introduction), nitrate showed itself to be a considerably worse nitrogen source than ammonium. About two-thirds of the isolates could not on the whole metabolise nitrate and the nitrate users exhibited, generally, a clear preference for ammonium. Only for five isolates, namely, *B. elegans, B. granulatus* (C 19), *B. luteus* (C 243), *B. subtomentosus* (C 30) and *Tricholoma pessundatum,* was nitrate equivalent to ammonium as a nitrogen source.

As may be gathered from the Introduction, the ability to utilise nitrate appears not to be of use as a phylogenetic criterion; the present results do not contradict this. In the genera *Boletus, Rhizopogon* and *Tricholoma* there were thus both nitrate users and non-nitrate users, and divergency could even be observed between isolates of the same species, as was, for instance, the case for *B. granulatus, B. subtomentosus* and *B. variegatus.* (On the other hand, isolates of *Amanita muscaria, B. bovinus, B. luteus* and *Paxillus involutus* behaved uniformly between themselves whether they were nitrate users or not.) In respect of ecology, however, a tendency towards dissimilarity could be observed, in that about half of the mycorrhiza-formers, but only one of the seven isolates which were designated litter-decomposers, viz. *B. subtomentosus* (C 30), were nitrate users. In this context Cochrane's (1958, p. 243) remark about non-nitrate users is appropriate, namely that their ability to survive in nature seems to be determined rather by their ecological situation than by their taxonomic position.

Nitrite nitrogen

In the cases in which one of the investigated substances could not be utilised as a nitrogen source, new mycelium was nevertheless formed on the surface of the inoculum. This, however, was not the case when nitrite was investigated, since the inoculum appeared in all cases to be quite sterile. This toxic effect of nitrite on higher fungi is well known and in forestry practice stump treatment with a 10 per cent solution of sodium nitrite is recommended as a protection against invasion of the stump by *Fomes annosus,* the root rot fungus. Rawald's (1963) discovery that *Tricholoma nudum* could to some extent utilise nitrite is noteworthy, but no parallel result was obtained in the present investigation.

Amide nitrogen

Of the two amides included in the experiment, acetamide and asparagine, the former will mainly be discussed here.

There is very little information in the literature as to the ability of fungi to utilise amides other than urea and those included in the group amino acids; for mycorrhiza-formers only Rawald's (1963) work is available. The result of his investigation on both mycorrhiza-forming and litter-decomposing *Tricholoma* species showed that propionamide was an excellent nitrogen source, equivalent to ammonium and asparagine, while valeramide produced somewhat poorer growth. A similarly positive answer was not obtained for acetamide in the present investigation, since about half of the isolates were quite incapable of making use of this nitrogen source. (This difference in results may be caused by Rawald's having autoclaved his nitrogen sources and perhaps having thereby split the amides). For five isolates, viz. *Agaricus campestris, Boletus elegans, B. subtomentosus* (C 30), *Lactarius deliciosus* and *Tricholoma pessundatum*, however, acetamide was quite as good a nitrogen source, or a better, than ammonium and asparagine. No correlation could be shown between the ability to utilise acetamide and asparagine. In point of ecology there was, however, a tendency for the litter-decomposers to have a somewhat greater possibility to make use of amide nitrogen than the mycorrhiza-formers.

Amine nitrogen

Diethylamine and D-glucosamine were investigated as amine nitrogen sources, and of these the latter was of especial interest, since its acetyl derivative is the basic unit of chitin, the cell-wall material of most fungi. The investigation by Rawald referred to above showed that most of his isolates were clearly stimulated by triethylamine. In this case, too, the lack of agreement between the results was striking (cf. amide nitrogen), since only two isolates in the present investigation, viz. those of *B. elegans* and *T. pessundatum*, gave a clearly positive growth response for amine nitrogen and then only for D-glucosamine. Further isolates showed more or less significantly that they could utilise amine nitrogen, without it therefore being necessary to regard this nitrogen source as acceptable to them. The result for *T. pessundatum* on D-glucosamine is remarkable, since the mycelium production was of the same order of magnitude as that with ammonium and

asparagine, which might indicate that this isolate is better adapted to making use of break-down products of chitin than many others. An investigation carried out independently of this experimental series showed, furthermore, that D-glucosamine was almost as good a carbon source for the fungus as glucose.

Amino acids

As was mentioned above, neither proline nor pyridine could be utilised as a nitrogen source, the latter substance being inadequately included under this heading, but like proline, a heterocyclic nitrogen compound. By contrast, the aliphatic amino acids glycine and L-asparagine were considerably more suitable. The results showed, however, a very heterogeneous pattern. For some isolates both amino acids were useless as a nitrogen source. Other isolates could make use of asparagine but not glycine while the situation might occasionally be reversed. It was, however, most usual for both amino acids to be utilisable, but then, as a rule, with a preference for asparagine, which might indicate that nitrogen in the amide position is more suitable as a nitrogen source than that bound as an amine.

The result for *Amanita citrina*, *A. muscaria* (C 4), *Boletus aeruginascens* and *B. badius* (two isolates) on asparagine should, however, be mentioned separately. After three weeks' culturing the inoculum of these isolates was apparently quite sterile, which indicates that asparagine may in some cases have an inhibiting effect resembling that of nitrite (see p. 36). This somewhat remarkable result is parallel with that obtained by Melin (1963) for *Boletus versipellis* and *Lactarius rufus* on glutamic acid. While many species showed optimum growth on this amino acid at concentrations of 5—25 mmol/l, the corresponding value for these two species was 0.05 mmol/l and above this concentration growth was markedly inhibited. By way of comparison it might be mentioned that the concentration of asparagine in the present study was 5.4 mmol/l. The tolerance amongst different species for a given compound can thus vary within wide limits, which implies that it is not possible to assess a substance as a nitrogen source before it has been tested in a concentration series. Negative results both in this and in most earlier investigations must therefore be treated with reserve.

The results reported above have mainly been discussed from the point of view of the nitrogen source. If, however, the fungus is con-

sidered instead, it may be stated that marked differences existed between different species. Some could only utilise one or a few of the nitrogen sources tested, while others exhibited a considerably wider spectrum and could make use of several, as for instance, was the case with *Boletus elegans* and *Tricholoma pessundatum*. This might also be so for isolates of the same species. Thus the one isolate of *B. granulatus* was a nitrate-user but not the other and there were also differences between them in respect of the ability to utilise glycine and L-asparagine. This difference was still more notable in the case of *B. subtomentosus,* but not, however, surprising, in view of the marked differences earlier shown by the isolates (Cap. III).

In summary, therefore, it may be stated that there was clear heterogeneity in the fungi studied, in respect of the ability to make use of the nitrogen sources available, and that this could also apply to isolates of the same species. In general terms, ammonium tartrate and L-asparagine were the two nitrogen sources which could best be utilised, as also to some extent glycine, acetamide and potassium nitrate. In contrast, D-glucosamine (with the exception of *Boletus elegans* and *Tricholoma pessundatum*), diethylamine, L-proline and pyridine were almost completely unusable as nitrogen sources, as was also potassium nitrite, which throughout showed a purely toxic effect on the fungi. No divergence could be discerned between mycorrhiza-formers and litter-decomposers, other than an indication that the former were more able to make use of nitrate nitrogen and the latter of amide nitrogen.

Chapter V. Utilisation of raw humus as a nitrogen source by mycorrhiza-forming and litter-decomposing fungi

A. Introduction

Of the large quantity of nitrogen which often occurs in podzolic soils under coniferous stands, that in the raw humus is the most interesting from the mycorrhizal point of view. A number of investigations (Harley, 1940; Werlich & Lyr, 1957; Mikola & Laiho, 1962; Lyr, 1963b and others) have demonstrated that the intensity of mycorrhiza (number of mycorrhizal points per unit volume) is decidedly largest in the upper parts of the humus layer, even if an abundant occurrence of mycorrhiza is reported from the mineral soil (Mikola *et al.*, 1966). The main reason for this unequal distribution is probably to be found in the fact that primarily the raw humus layer is supplied with nutrient elements by water percolating from the canopy and through leaching from the litter (cf. Tamm, 1951; Nykvist, 1963). Furthermore, the nitrogen in the humus layer is more easily mineralised than that in the inorganic horizons (Tamm, 1962; Tamm & Pettersson, 1969).

Only a fraction of the total nitrogen in the humus is, however, available to higher plants while the greater part exists in organically bound form, in part probably as complex compounds with lignin and phenolic substances (Bremner, 1965). By hydrolysing the material with mineral acids it is possible to obtain general information about the chemical nature of the form of the bound nitrogen. Nömmik (1967) showed by such a method that 50 per cent of the total nitrogen in raw humus consisted in amino acid N, 11 per cent of hexosamine N, 15 per cent of ammonium N and six per cent of unidentified N, while the remainder formed a fraction of non-hydrolysable N. In other studies (reviewed by Bremner) it has been possible to identify after hydrolysis other nitrogen forms, such as aliphatic amines and derivatives of purine and pyrimidine, but this appears not to make up more than about one per cent of the total nitrogen in the humus.

So that the nitrogen bound in the humus may be utilised by higher plants, it must first be mineralised, which takes place by the agency of microorganisms capable of producing extra-cellular enzymes (Alexander, 1961). Simultaneously, there is, however, a microbial

fixing of nitrogen—the immobilisation of nitrogen. This implies that the mineralisation must be of greater extent than the immobilisation if there is to be a net mineralisation and hence a surplus of nitrogen which can be made use of by the plants. The so-called internal nitrogen cycle, which arises as a result of the two processes involves, however, only an active part of 10—15 per cent of the organically bound nitrogen, represented by readily turned over material, while the remainder, a more inactive part, consisting of stabilised residues and humus compounds, seems mainly to lie outside the internal cycle (Jansson, 1958).

Many kinds of soil microorganism, from bacteria to the higher fungi, are considered to play an active part in the mineralisation, and in forest soil with its relatively low pH, the fungi are probably prominent. The function of the mycorrhiza-forming fungi in this respect is uncertain, but an attempt will be made to elucidate it in this and the subsequent chapter.

B. Material and methods

1. Preparation of N^{15} – labelled raw humus

Raw humus was collected in a pine stand at Bogesund near Stockholm and roots and coarser undecayed plant material were carefully removed. The dry matter content of the cleaned humus was ca 40 per cent in air dried samples. To every batch of 1 kg cleaned humus was added 375 mg ammonium sulphate with 54 atomic-% N^{15}-excess and further, 5 g of glucose (Nömmik, pers. comm.) for activating the microflora. The quantity of ammonium sulphate added was calculated to give the humus nitrogen an approximate N^{15}-excess of one per cent, calculated on the basis of air-dried material. To obtain as homogeneous a mixture of the chemicals as possible, they were scattered into the humus while it was in an electric household mixer (Electrolux) in action. The prepared portions of humus were mixed together in a black plastic sack, which was sealed. Air exchange was made possible by the insertion of a coarse tube, one end of which was covered with a well perforated plastic membrane. The sack was stored at room temperature for six months, after which the humus was dried at 40°C in an oven with a fan. To eliminate as far as possible that part of the labelled nitrogen which after six months had not been incorporated in the humus in organically bound form, but which still existed as exchangeable nitrogen, portions of 200 g of the dry humus were shaken together with 1000 ml 0.05 M sulphuric acid for three hours. After

Table 4. Content of total N and inorganic N (expressed on the basis of oven-dry weight) and N^{15}-excess (atomic-%) in fertile raw humus both before and at various times after the addition of N^{15}-labelled ammonium sulphate.

Time of sampling	Total N		Inorganic N			
			Ammonium		Nitrate	
	%	N^{15}-excess, atomic-%	ppm	N^{15}-excess, atomic-%	ppm	N^{15}-excess, atomic-%
Before addition of $(N^{15}H_4)_2SO_4$	1.3		25		8	
Immediately after addition	1.3	0.814	226	39.16	7	
35 days after addition			317	7.932	3	
66 days after addition			472	5.775	7	
111 days after addition			743	4.124	62	
180 days after addition	1.2	0.805	1099	3.328	136	3.298
After treatment with 0.05 M sulphuric acid and washing			39	1.891	5	0.702
After gamma-irradiation	1.2	0.569	143	2.420	15	0.894

filtration and rinsing with aq. dest. (1000+500+500+500 ml per portion) in a Büchner funnel the humus was once more dried at 40°C in the same oven. When thoroughly dry it was sieved through a sieve of 2.0 mm mesh, placed into plastic containers holding 40.0 g each and was sterilised by gamma radiation (2.5 megarads) by Radona Irradiation AB, Skärhamn, Sweden.

The incorporation of the labelled nitrogen into the humus was followed by means of serial analyses of, amongst other things, the exchangeable ammonium nitrogen; the results obtained are shown in Tab. 4.

2. Preparation of petri dishes

For the experiment 14 mm tall plastic petri dishes 9 cm in diameter and having an 8 mm high separating wall in the middle (Falcon Plastics) were used, see Fig. 6. The area of each half of the dish was 27.2 cm^2.

As culture medium in one half of the dish was used a "glucose agar" of the following composition:

Agar powder	7.5 g
Glucose	5.0 g
KH_2PO_4	500 mg
$MgSO_4 \cdot 7\ H_2O$	250 mg
Ferric citrate (1 %)	0.25 ml
$MnSO_4 \cdot 7\ H_2O$ (0.2 %)	0.25 ml
$ZnSO_4 \cdot 7\ H_2O$ (0.02 %)	0.25 ml
Thiamine	25 μg
Folic acid	50 μg
Biotin	5 μg
HCl (0.1 M)	2.0 ml
Aq. dest.	500 ml

Every portion was weighed in a 1000 ml Erlenmeyer flask and to obtain a homogeneous solution, the mixture was heated in a water bath while being shaken, until it was clear; it was then autoclaved at 1 atm. for 15 minutes. During this procedure the glucose agar solution decreased in weight by ca 20 g. After it had cooled to 40°C it was poured, forming a slight heap, into one half of each petri dish. One portion sufficed for 20 petri dishes, so that each half-dish contained ca 24 ml. Since the agar powder (Difco), with a total nitrogen content of 0.12 per cent, was the only nitrogen source in the glucose agar (the amount of nitrogen in the added vitamins was quite negligible), the nitrogen content per half-dish was estimated at ca 0.4 mg. This value was confirmed by analysis of prepared dishes.

As culture medium in the other half of the dish a "humus agar" of the following basic composition was used:

Agar powder	7.5 g
KH_2PO_4	500 mg
$MgSO_4 \cdot 7\ H_2O$	250 mg
Ferric citrate (1 %)	0.25 ml
$MnSO_4 \cdot 7\ H_2O$ (0.2 %)	0.25 ml
$ZnSO_4 \cdot 7\ H_2O$ (0.02 %)	0.25 ml
Aq. dest.	500 ml

After the agar had been melted and autoclaved as described above, the flask was placed in a water bath at 39—40°C attached to a magnetic stirrer. When the contents of the flask had attained the temperature of the water bath, 40 g of slightly warmed (50°C) gamma-sterilised, N^{15}-labelled raw humus (see above), 500 mg of heat-sterilised, pulverised calcium oxide and a sterile magnetic rod were added. The mixture was then vigorously stirred for one hour, after which it was

poured in to the empty half of the petri dishes. The humus agar solidified almost instantly in the dishes, which prevented sedimentation of the humus particles. The humus agar, like the glucose agar, had a pH of 4.9.

Each dish was inoculated with two rectangular pieces of inoculum (ca 3 × 6 mm), which were placed at right-angles across the separating wall about 3 cm from each edge of the dish. The controls were inoculated with pieces of malt agar without mycelium. The dishes were incubated at 25°C in darkness for 21 days. A planimeter was used to measure the mycelium-covered area on both the glucose agar and the humus agar halves of the dishes. Furthermore, the mycelium density was ocularly estimated. To avoid contamination of the glucose agar half by humus from the humus agar half, the latter was removed before the glucose agar was dried in an oven at 40°C. When thoroughly dry the glucose agar with mycelium could easily be removed from the dish. In order to obtain a sufficient quantity of nitrogen for analysis of the N^{15}-excess, two glucose agar halves were united into one sample, which was analysed for the amount of total N and N^{15}-excess.

3. Nitrogen analyses

Total N was determined by the micro-Kjeldahl procedure, using copper and selenium as catalysts. The ammonia liberated was distilled over into a receiver with 4 per cent boric acid with indicator added (4 ml indicator solution—12.5 mg methylene blue+27 mg methyl red in 30 ml ethanol—per litre of boric acid solution), and the quantity was determined by titration with hydrochloric acid.

Exchangeable ammonium and nitrate N were extracted from the humus using 1 M potassium chloride. The extract was distilled in the presence of borate buffer (pH 8.8) and the liberated quantity of ammonia was determined by titration. For the residue after distillation, nitrate was determined by means of Devarda's alloy.

The nitrogen isotope ratio was determined as the atomic-% N^{15}-excess after Rittenberg's method (1946) by means of a Consolidated Nier isotope ratio mass spectrometer (Model 21-202).

C. Results and discussion

The method employed was so designed that the growth conditions for the mycorrhiza-forming fungi resembled to some extent those in nature. The glucose agar was therefore considered to represent the root, to

which the fungi must have recourse for obtaining their energy requirement; since they themselves, in contrast to the litter-decomposers, have either none or a very limited enzyme complement for this purpose (see Cap. III). The humus agar half was, of course, intended to correspond to the raw humus, which forms the nitrogen and mineral nutrient source for the fungi. The method was based on the principle that an increase in the amount of nitrogen in the glucose agar half after the inoculation of the dish and the growth of the mycelium, would reflect the capacity of the inoculated fungus for utilising the nitrogen sources available to it in the humus agar. To increase the chances of assessing this, the investigation was carried out with N^{15}-labelled raw humus.

The course of the immobilisation of the added labelled ammonium sulphate in the raw humus was followed by means of serial analyses (Tab. 4). The results of these showed that the supplied nitrogen was rapidly incorporated into the humus as organically bound nitrogen. The N^{15}-excess in the exchangeable NH_4-N thus decreased from ca 40 per cent to seven per cent in 35 days until after 180 days it was slightly more than three per cent. At the same time as the immobilisation, there was also a mineralisation, which resulted in an increase in the exchangeable NH_4-N, from 226 ppm immediately after the addition of ammonium sulphate, to 1099 ppm and in the exchangeable NO_3-N from 7 ppm to 136 ppm. This pattern agrees well with that obtained by Nömmik (1968). By treatment of the humus with 0.05 M sulphuric acid, and subsequent washing, the majority of the exchangeable N was removed. The gamma sterilisation which followed caused, however, an increase of the order of 3.5 times. A sterilisation effect of this kind was earlier noted by Eno & Popenoe (1963), Bowen & Cawse (1964), Coleman & Mcfadyen (1966) and Rovira & Bowen (1969), and appears to be caused both by the partial deamination of the amino acids and proteins (Maxwell *et al.*, 1954; Sharpless *et al.*, 1955 and Bennett & Garrison, 1959) and by the fact that some enzymes, including urease, are not inactivated by radiation (McLaren *et al.*, 1957, 1962). A preliminary experiment with different radiation doses showed that 2.5 megarads was adequate for giving total sterilisation; this was checked by inoculating irradiated humus particles on dishes containing malt agar and peptone agar. This result was in good agreement with earlier experience (Bowen & Cawse, 1964).

As the experiment with the glucose/humus agar dishes was laid out, it was not possible to determine the absolute mycelium production. However, in order to give an impression of this, in Tab. 5 are shown

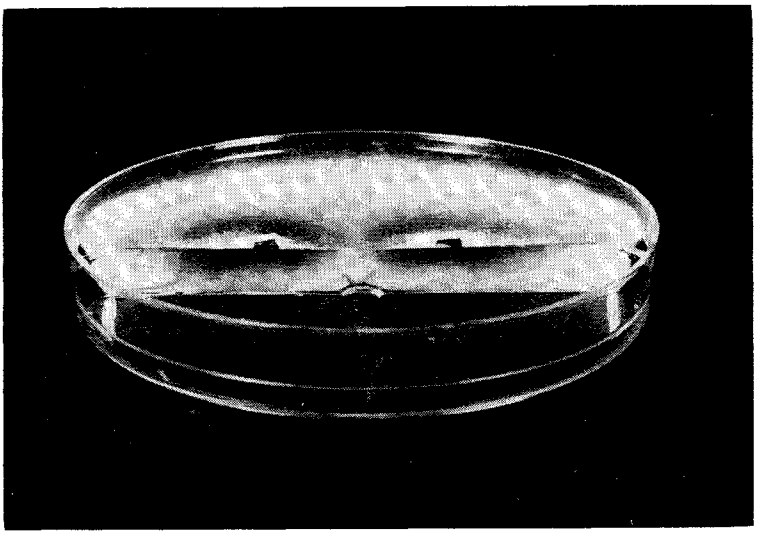

Fig. 6. Glucose/humus agar dish inoculated with *Boletus variegatus* (C 35) after three weeks' incubation in darkness at 25°C. The humus agar half was removed before the dish was photographed. Observe the submerged mycelium in the glucose agar.

both the size of the area covered by the mycelium and an estimate of the density of the mycelium mat. It should be mentioned that the mycelium also grew down into the agar (Fig. 6) and occasionally faster there than on the surface, which was easy to observe on the glucose agar half. The extent of the submerged mycelium on the humus agar side could not be observed; hence the values given for the area of the mycelium mat concerned in this case the situation on the surface of the humus agar.

In general terms, the inoculation resulted in a satisfactory production of mycelium and only *Boletus aeruginascens* developed poorly. In terms of area, matters were the same for *B. subtomentosus* (C 118), but this isolate produced a thick mycelium in the form of a white cushion, which reached the lid of the petri dish. The mycelium mat, which in most cases was denser on the humus agar side, was most usually equally extended on both halves of the agar or with some preference for the humus agar side (Fig. 7). On some occasions, however, there were marked deviations, as in the case of the two isolates of *B. variegatus*, which covered almost all of the humus agar half but only one-third of the glucose agar half. The situation with *B. granulatus*

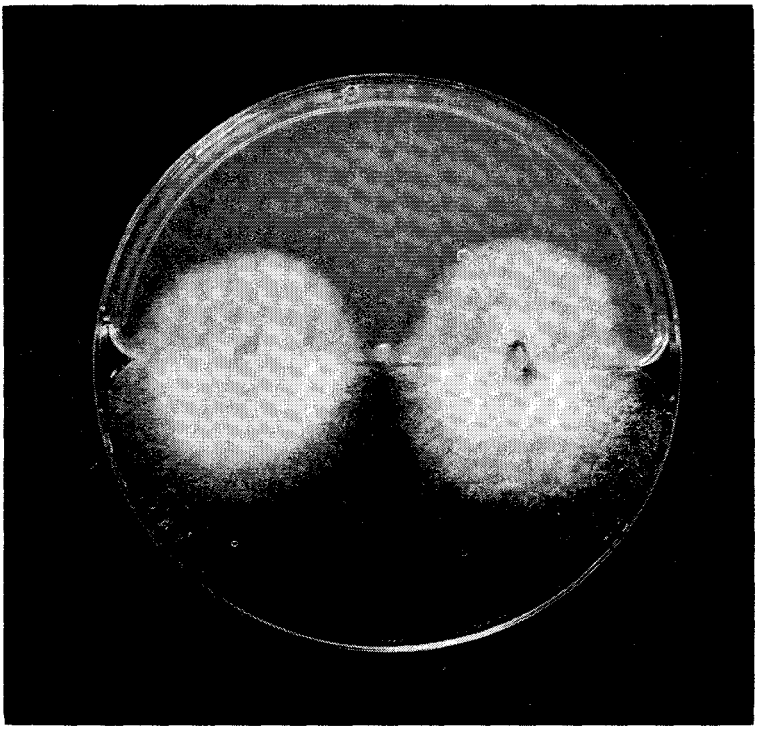

Fig. 7. Glucose/humus agar dish inoculated with *Corticium bicolor* after three weeks' incubation in darkness at 25°C.

(C 240) and *B. bovinus* (C 15) was similar, while that for *Agaricus campestris* and *B. edulis* (C 18) was reversed; these covered all or a large part of the glucose agar half, but only a small part of the other half.

The results of the analysis of the glucose agar halves for total N and atomic N^{15}-excess are shown in Tab. 5. To give a more comprehensible picture of the results, the average values for each isolate in respect of the N^{15}-excess, expressed in micrograms, are presented in graphical form, too (Fig. 8). In all cases, with the exception of the uninoculated controls, it was found that labelled N had been transferred from the humus agar half to the glucose agar half, and by reference to the content of N^{15}-excess in the glucose agar, the material could be divided into three groups. In the first group, comprising 24 isolates of mycorrhiza-formers, five isolates of fungi of unknown ecological type and five isolates of litter-decomposers, a maximum N^{15}-excess of

Table 5. Mycelium growth on glucose/humus agar dishes and amount of total N and atomic N^{15}-excess in glucose agar including mycelium after three weeks' incubation at 25°C. Each value represents two dishes united into one sample. The fungal isolates have been classified by ecological type. M = ectotrophic mycorrhiza-formers, L = litter-decomposers and — = fungi of unknown ecological type.

Species	Type	Mycelium growth cm^2 on glucose-half	Mycelium growth cm^2 on humus-half	Estimated mycelium mat density on glucose-half	Estimated mycelium mat density on humus-half	Analyses of glucose-half incl. mycelium N_{tot} mg	Analyses of glucose-half incl. mycelium N^{15}-excess atomic %	Analyses of glucose-half incl. mycelium N^{15}-excess μg
Agaricus campestris C 267	L	54.4 54.4	10.6 9.6	medium	thick	2.59 2.77	1.272 1.342	32.9 37.2
Amanita citrina C 274	M	38.5 50.1	34.6 48.5	medium-thick	medium	1.05 1.26	0.146 0.187	1.5 2.4
Amanita muscaria C 4	M	13.0 13.7	7.4 6.7	medium	thick	1.23 1.30	0.219 0.267	2.7 3.5
Amanita muscaria C 256	M	21.8 20.4	35.9 29.1	thin	thick	1.23 1.37	0.266 0.177	3.3 2.4
Amanita rubescens C 6	M	26.8 40.4	33.0 40.3	thin	thin-medium	0.91 1.05	0.199 0.136	1.8 1.4
Boletus aeruginascens C 264	—	5.9 8.2	4.4 9.1	thin	thin-medium	1.12 1.23	0.144 0.216	1.6 2.7
Boletus badius C 251	M	23.4 22.8	18.8 20.6	thin-medium	medium	1.02 1.30	0.317 0.193	3.2 2.5
Boletus bovinus C 15	M	25.8 24.0 25.1	54.4 54.4 41.3	medium	thick	1.19 0.88 1.02	0.156 0.151 0.307	1.9 1.3 3.1

Table 5. ***(continued)***

Species	Type	Mycelium growth cm² on glucose-half	Mycelium growth cm² on humus-half	Estimated mycelium mat density on glucose-half	Estimated mycelium mat density on humus-half	Analyses of glucose-half incl. mycelium Ntot mg	N^{15}-excess atomic %	N^{15}-excess μg
Boletus		32.7	37.1	medium	thick	1.09	0.095	1.0
bovinus	M	30.5	37.6			0.91	0.087	0.8
C 16								
Boletus		38.0	3.5	thin	thin	1.09	0.137	1.5
edulis	M	39.7	3.9			1.05	0.076	0.8
C 18								
Boletus		31.9	15.2	thin	thin	1.02	0.155	1.6
edulis	M	15.7	4.5			1.23	0.066	0.8
C 93								
Boletus		13.7	17.7	medium	medium	0.98	0.067	0.7
elegans	M	10.3	11.8			1.16	0.089	1.0
C 117								
Boletus		49.7	54.4	thick	medium	1.05	0.191	2.0
granulatus	M	50.5	54.4			1.19	0.264	3.1
C 19		51.7	54.4			1.23	0.193	2.4
Boletus		16.2	54.4	medium	thin-	0.88	0.035	0.3
granulatus	M	26.5	51.1		medium	0.95	0.097	0.9
C 240								
Boletus		26.0	40.0	medium	thick	1.02	0.072	0.7
luteus	M	26.1	46.4			1.05	0.063	0.7
C 81								
Boletus		39.1	51.0	medium-	thick	0.98	0.163	1.6
luteus	M	36.2	50.8	thick		1.09	0.199	2.2
C 243								
Boletus		50.0	50.3	thin	medium	1.12	0.074	0.8
piperatus	—	50.7	48.9			—	0.083	—
C 277								

Table 5. ***(continued)***

Species	Type	Mycelium growth cm² on glucose-half	Mycelium growth cm² on humus-half	Estimated mycelium mat density on glucose-half	Estimated mycelium mat density on humus-half	Analyses of glucose-half incl. mycelium N_{tot} mg	Analyses of glucose-half incl. mycelium N^{15}-excess atomic %	Analyses of glucose-half incl. mycelium N^{15}-excess μg
Boletus		54.4	54.4	medium	medium-	1.33	1.059	14.1
subtomentosus	L	54.4	54.4		thick	1.23	1.025	12.6
C 30		54.4	54.4			1.40	1.107	15.5
		54.4	54.4			0.95	0.954	9.1
Boletus		8.9	3.9	thick	thick	1.33	0.346	4.6
subtomentosus	M	6.1	3.3			1.47	0.625	9.2
C 118								
Boletus		17.9	49.6	medium	medium-	0.91	0.039	0.4
variegatus	M	19.5	49.9		thick	0.91	0.032	0.3
C 35		20.3	51.6			0.98	0.032	0.3
Boletus		15.3	51.9	thin	medium	1.02	0.084	0.9
variegatus	M	14.3	51.6			1.02	0.019	0.2
C 79								
Cenococcum		18.7	19.0	medium-	thick	0.95	0.118	1.1
graniforme	M	11.0	12.0	thick		0.91	0.138	1.3
D 10								
Clitocybe		31.7	54.4	thin	medium	1.05	—	—
nebularis	L	32.3	54.4			1.05	0.064	0.7
C 137								
Clitopilus		50.8	54.4	medium-	medium-	0.98	0.192	1.9
prunulus	—	51.8	54.4	thick	thick	0.91	0.164	1.5
C 45		48.4	54.4			0.88	0.212	1.9
Corticium		18.9	29.8	thick	thick	1.02	0.080	0.8
bicolor	M	18.0	31.5			1.23	0.137	1.7
C 269		16.8	25.9			1.37	0.094	1.3
Fomes		54.4	54.4	thin	medium	1.02	0.748	7.6
annosus	L	54.4	54.4			1.05	0.853	9.0
Sä 1		54.4	54.4			0.98	0.597	5.9

Table 5. ***(continued)***

Species	Type	Mycelium growth cm² on glucose-half	Mycelium growth cm² on humus-half	Estimated mycelium mat density on glucose-half	Estimated mycelium mat density on humus-half	Analyses of glucose-half incl. mycelium Ntot mg	N^{15}-excess atomic %	N^{15}-excess μg
Hebeloma		16.7	32.7	thin-	medium-	1.02	0.056	0.6
crustuliniforme	M	18.1	26.2	medium	thick	1.12	0.058	0.6
C 87		21.5	22.2			1.16	0.051	0.6
Hygrophoropsis		45.9	35.4	thin-	thin-	0.88	0.156	1.4
aurantiaca	L	47.3	42.3	medium	medium	0.91	0.153	1.4
C 135								
Hygrophoropsis		54.4	52.3	thin	thin	1.19	0.455	5.4
aurantiaca	L	54.4	50.7			1.16	0.379	4.4
C 245								
Lactarius		16.0	9.0	medium	thin	1.33	0.487	6.5
deliciosus	M	15.1	4.9			1.54	0.414	6.4
C 154								
Paxillus		31.8	13.8	medium	medium-	0.91	0.230	2.1
involutus	—	29.0	16.1		thick	0.88	0.159	1.4
C 122		46.5	22.1			0.98	0.172	1.7
Paxillus		32.3	32.4	thin-	medium-	1.09	0.091	1.0
involutus	—	34.6	25.0	medium	thick	1.19	0.101	1.2
C 248								
Pholiota		54.4	54.4	medium	medium	0.95	0.030	0.3
mutabilis	L	54.4	54.4			0.88	0.040	0.4
C 262		54.4	54.4			0.98	0.061	0.6
Rhizopogon		19.8	23.0	thin-	medium	0.95	0.059	0.6
luteolus	M	22.3	15.8	medium		1.02	0.128	1.3
C 282								

Table 5. ***(continued)***

Species	Type	Mycelium growth cm² on glucose-half	Mycelium growth cm² on humus-half	Estimated mycelium mat density on glucose-half	Estimated mycelium mat density on humus-half	Analyses of glucose-half incl. mycelium Ntot mg	Analyses of glucose-half incl. mycelium N^{15}-excess atomic %	Analyses of glucose-half incl. mycelium N^{15}-excess µg
Rhizopogon		39.0	43.4	medium	medium-	0.98	0.260	2.5
roseolus	M	44.4	40.5		thick	1.12	0.250	2.8
C 236		42.1	42.4			1.12	0.268	3.0
Stropharia		53.9	42.3	medium	thick	1.05	0.167	1.8
aeruginosa	L	54.4	38.8			1.09	0.145	1.6
C 265								
Tricholoma		9.9	18.9	thin	medium-	0.88	0.080	0.7
albobrunneum	M	10.2	20.9		thick	0.81	0.060	0.5
C 278		11.8	16.6			0.91	0.109	1.0
Tricholoma		20.4	19.7	thin	medium	1.33	0.081	1.1
imbricatum	M	20.0	20.1			1.16	0.151	1.8
C 223								
Tricholoma		52.5	37.2	thin	medium	1.16	0.124	1.4
nudum	L	52.3	45.0			1.12	0.102	1.1
C 225								
Tricholoma		49.0	54.4	medium	medium	0.91	0.146	1.3
pessundatum	M	49.6	54.4			0.88	0.156	1.4
C 226		45.0	38.7			1.02	0.150	1.5
Control inoculated						1.02	0.424	4.3
with pure malt agar						1.12	0.487	5.5
Control						0.95	0.005	<0.1
uninoculated						0.88	0.004	<0.1

0.317 per cent was obtained. In the second group, consisting of two mycorrhiza-formers—*Boletus subtomentosus* (C 118) and *Lactarius deliciosus*—and one litter-decomposer—*Hygrophoropsis aurantiaca* (C 245)—, the excesses were of the same order as for the control dishes "inoculated" with pieces of malt agar lacking mycelium, viz. on the average ca 0.450 per cent, while the values for the third group were higher, and varied between 0.700 and 1.300 per cent. In the last group there were three isolates—*Agaricus campestris, Boletus subtomentosus* (C 30) and *Fomes annosus*—which were all designated as litter-decomposers.

The high percentage of N^{15}-excess in the control dishes "inoculated" with pure malt agar must have been caused by a transfer of labelled nitrogen from the humus agar to the pieces of "inoculum" during the three weeks in which the dishes were incubated, since the result from the uninoculated dishes showed that no transport could take place via the plastic wall separating the halves of the dish. Whether the labelled nitrogen had been transferred via the pieces of inoculum to the glucose agar or whether it had accumulated only in them, was not apparent from the experiment, since both components were analysed as a whole. The fact that the percentage of N^{15}-excess for the first group of isolates was lower than that for the controls must necessarily depend on influence from the fungus. Since the mycelium production, as mentioned above, was apparently greater on the humus agar side than on the glucose side, it is to be supposed that the labelled nitrogen which in the controls was transferred to the pieces of inoculum, was immediately utilised by the fungus, in the main for producing mycelium on the humus agar side. In support of this interpretation is the fact that there was a tendency for the percentage of N^{15}-excess to be lower on the glucose agar side when the area covered by mycelium on that side was small relative to the area on the humus agar side.

It was not possible to determine how large an amount of the labelled nitrogen was transferred from the humus agar half to the glucose agar half via the mycelium and the pieces of inoculum, respectively. A comparison between the results for the first group and the third indicated, however, that there was a fundamental difference between them. Thus in the first, two micrograms of labelled nitrogen, on the average, were found in the glucose agar half, while the corresponding value for *Agaricus campestris, Boletus subtomentosus* (C 30) and *Fomes annosus* in the third group was 35, 13 and 8 micrograms, respectively. It seems probable that this difference was caused by the fungi in the two groups having possessed differing ability to utilise

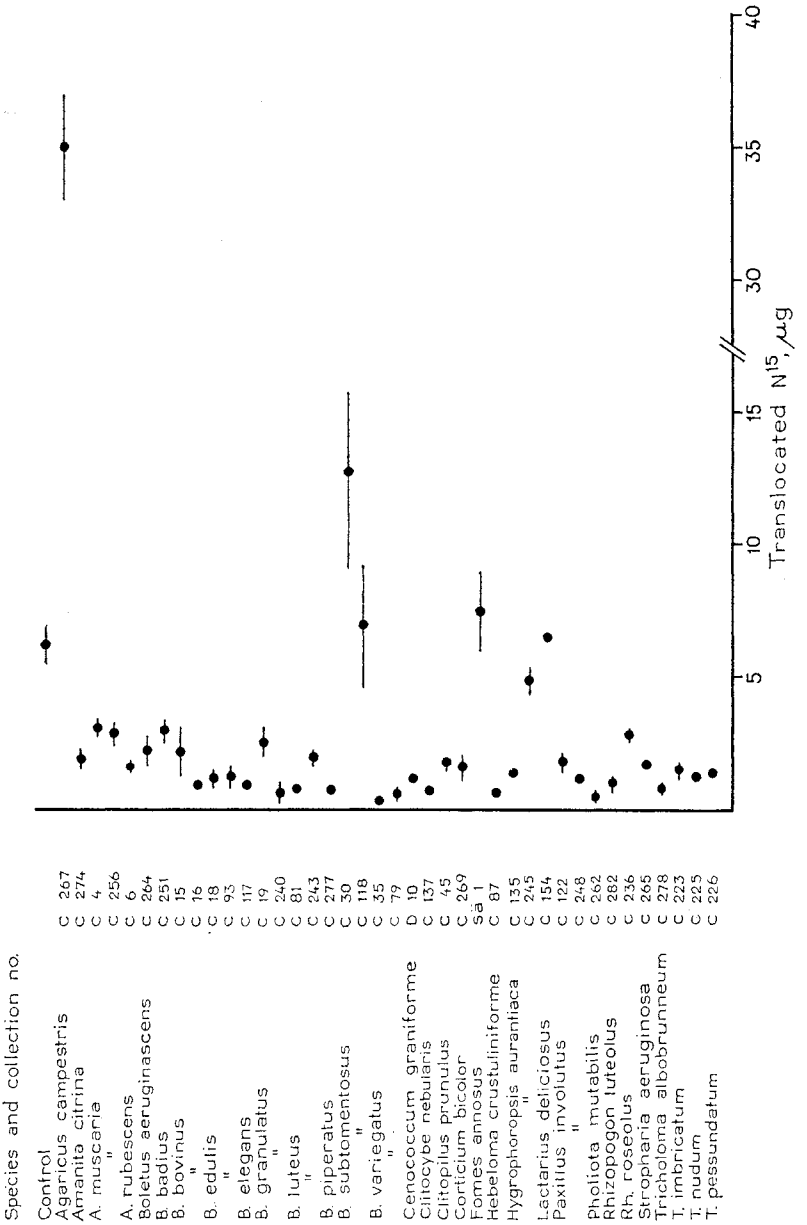

Fig. 8. Amount of labelled nitrogen translocated from the humus agar half to the glucose agar half in glucose/humus agar dishes inoculated with mycorrhiza-formers and litter-decomposers after three weeks' incubation in darkness at 25°C. The control was "inoculated" with pieces of pure malt agar only. The points give the average values and the horizontal bars the maximum and minimum values. For further information, see Tab. 5 and the text.

that nitrogen source available. If this was the case, the result indicates that the fungi in the first group were directed solely to the exchangeable nitrogen in the raw humus or that they could also use the organically bound nitrogen, but to so limited an extent that the mineralised nitrogen was immediately immobilised by the production of mycelium on the humus side; while the fungi in the third group were able to mineralise so much nitrogen that not all was used up on the humus agar side but some was transferred to the glucose agar side. Since nitrogen mineralisation is a consequence of the microorganisms' production of extra-cellular enzymes, it was justifiable to compare the results from this experiment with those from the enzyme investigation (Cap. III). Seventeen of the 34 isolates in the first group were studied in respect of their production of cellulase, pectinase, proteinase and laccase. Thirteen of these showed throughout a low enzyme potential, while *Clitopilus prunulus* produced cellulase and proteinase, *Hygrophoropsis aurantiaca* (C 135) produced cellulase and pectinase, *Tricholoma albobrunneum* pectinase and *T. nudum* all four enzymes, although here cellulase was present in scarcely demonstrable amounts. On the other hand, all three isolates in the third group had a comparatively high enzyme potential. *Agaricus campestris* and *Fomes annosus* thus produced all four enzymes investigated, while *Boletus subtomentosus* (C 30) produced three of them, viz. cellulase, pectinase and proteinase. However, this species did not form laccase or did so to a limited extent (Tab. 3).

The fairly good agreement between the behaviour of the isolates of both groups in the two investigations, indicates that all three of the isolates last mentioned were able to utilise the organically bound nitrogen in the humus. In contrast to the production of cellulase and pectinase, that of proteinase and laccase may be considered to affect nitrogen mineralisation, since the nitrogen in the raw humus is supposed to be bound up, for instance, in proteins and in the lignin complex. It was, however, not possible to find any correlation between the extent of the proteinase and laccase production which the fungi exhibited in the enzyme experiment, and their capacity for exploiting the bound humus nitrogen. In view of the fact that the experimental conditions were totally dissimilar in the two experimental series, this was not surprising, since it is well known that the environment can influence the extra-cellular enzyme production of a fungus. It is also conceivable that enzymes other than proteinase and laccase may have been decisive. This might explain why *Tricholoma nudum*, which exhibited high enzyme potential in respect of both of these enzymes,

gave rise to a negative result in the present experimental series. Another reason might be that this isolate had the ability to mineralise the humus nitrogen without itself being able to utilise the break-down products.

What possibility the three isolates forming the intermediate group had of utilising the bound nitrogen, is uncertain. The amount of labelled nitrogen recovered on the glucose agar side of dishes inoculated with one of these three was, in fact, as great as that in the control dishes. Undoubtedly, both *Hygrophoropsis aurantiaca* (C 245) and *Lactarius deliciosus* showed an enzyme activity higher than that of most of the isolates in the first group; but since the enzyme potential of *Boletus subtomentosus* (C 118) was very low, and since in the glucose agar of dishes inoculated with the other isolate of *H. aurantiaca* (C 135), only small amounts of N^{15} in excess could be found, despite the fact that both isolates were in very good agreement in the enzyme investigation, this question must be left open.

A summary of the results shows that all of the mycorrhiza-formers, and fully half of the litter-decomposers included in the experiment, seemed to be badly adapted for making use of the organically bound nitrogen in raw humus. A positive indication in this respect was obtained only for *Agaricus campestris, Boletus subtomentosus* (C 30) and *Fomes annosus*.

Chapter VI. Utilisation of raw humus as a nitrogen source by pine seedlings in the presence of mycorrhiza-forming and litter-decomposing fungi

A. Introduction

The preceding experimental series attempted to elucidate the capacity of the mycorrhiza-forming fungi for utilising the organically bound nitrogen in the raw humus. The results of this investigation, like those from the enzyme investigation reported in Cap. III (p. 22), and both investigations involving the fungi independently of roots, indicate, however, that they are poorly endowed for the task. It is not, however, possible to conclude that this is also the case when they are acting in intimate contact with the roots of plants, since the possibility cannot be ignored that these can influence the behaviour of the fungi by exuding substances which can induce enzyme production, in the same way as they, *de facto,* influence the growth of the fungi (Melin, 1954, 1959; Melin & Das, 1954). Other factors may also be involved.

To try to elucidate this question, a series of experiments was performed on the culturing together of pine seedlings and fungi, mainly mycorrhiza-formers. Two of these experiments are reported here.

B. Material and methods

In this experimental series, N^{15}-labelled raw humus of the preparation used in previous experimental series, was employed. After the extraction of exchangeable nitrogen, rinsing with distilled water, drying and sieving (see p. 41), a substrate of the following composition was prepared:

N^{15}-labelled humus	200 g
Sand	400 g
Perlite	100 g
Calcium oxide	3 g
Aq. dest.	400 ml

The sand had previously been washed in running water for at least 24 hours, had been dried and sieved, first through a sieve of 1.5 mm mesh and then through one of 0.5 mm mesh. The fraction which did not pass through the latter was used in the experiment. Perlite, which

completely lacks nutritive value, is a granular material of volcanic origin. The ingredients were mixed together in an electric household mixer for five minutes. The amount of calcium oxide added was so calculated that the humus mixture had a pH of 4.9. Clear plastic capsules (Cerbo plast, 105 ml) were filled with the substrate, 65 g per capsule; these were then closed with tightly fitting plastic lids and were gamma-sterilised by a dose of 2.5 megarads (p. 42). The humus mixture contained after sterilisation 0.34 per cent total nitrogen, with 0.57 atomic-% N^{15}-excess and 60 ppm exchangeable ammonium nitrogen with 2.14 atomic-% N^{15}-excess, based on the dry weight.

To produce material for inoculation, pure cultures of mycorrhiza-forming and litter-decomposing fungi were cultured in Erlenmeyer flasks on a nutrient solution identical with that given in Cap. IV (p. 29), to which had been added 1.0 g ammonium tartrate per litre. The inoculation method and culture conditions were identical with those in the above-mentioned chapter. Every Erlenmeyer flask was in addition provided with ca 20 glass beads. After three weeks' incubation, the nutrient solution was decanted and replaced with ca 25 ml of sterile solution of the same composition as that mentioned above, except that the concentration of ammonium tartrate had been reduced to 0.2 g per litre. The contents of the Erlenmeyer flasks were shaken vigorously for about a minute, after which the capsules of gamma-sterilised humus mixture were inoculated with 20 ml of the mycelium suspension thus obtained. One of the two experiments reported in this chapter was partly arranged as an experiment for joint culturing of two species of fungi. In this case the capsules were inoculated with 10 ml of suspension of each species. The inoculation was carried out through the plastic lid by means of a syringe with a coarse cannula, and the suspension was distributed as evenly as possible throughout the capsule. To permit air circulation, each capsule was fitted with a narrow tube filled with cotton wool. One end of the tube was drawn out to a capillary, which penetrated the lid of the capsule. Each treatment comprised five capsules.

A month after inoculation, the capsules were planted with sterilely grown seedlings of Scots pine *(Pinus silvestris* L.). Seeds from open pollination, collected in Gävleborg county, were sterilised in a calcium hypochlorite solution (10 g calcium hypochlorite in 140 ml water) for two hours and then placed on water agar petri dishes. After 10 to 14 days, seedlings suitable for planting had been obtained. A hole was made in the plastic lids of the capsules by means of a hot mounted needle; through this the root and a few millimetres of the hypocotyl

was introduced. The hole surrounding the hypocotyl was sealed with heat-sterilised, water-free lanoline.

The cultures were then transferred to a phytotron. The photoperiod employed was 16 hours, the illuminance was 30,000—40,000 lux and the day and night temperature were 20°C and 15°C, respectively. While the cultures were in the phytotron, they were watered when necessary with sterile distilled water and were inoculated once more with 20 ml of mycelium suspension. The amount of ammonium tartrate was on this occasion increased to 0.5 g/l. This was done about two months after the transfer of the capsules to the phytotron. After four months in the phytotron, the experiment was terminated and the plant material was analysed for the frequency of mycorrhiza, shoot and root dry weight and the shoots for amount of total nitrogen and N^{15}-excess.

The frequency of mycorrhiza was determined with the aid of a binocular microscope, and the material was divided into four groups, namely:

(a) plants lacking mycorrhiza-formation ("none")
(b) plants with scarce mycorrhiza-formation
(c) plants with moderate mycorrhiza-formation
(d) plants with abundant mycorrhiza-formation

having 0, 1—10, 11—50 and >50 per cent, respectively, of the short roots transformed to ectotrophic mycorrhiza. In many cases, and in particular when there was doubt as to whether mycorrhiza-formation was established, the roots were sectioned for microscopic examination. The sections were stained according to Jackson's (1947) method, with saffranin and aniline blue in picric acid. The occurrence of a Hartig net, together with a more or less developed mantle, were the criteria used in deciding whether an ectotrophic mycorrhiza was established.

Shoot and root dry weight were determined after the material had been dried at 60°C in an oven with a fan for 48 hours.

The total N and nitrogen isotope ratio were determined in the way described above (p. 44).

C. Results and discussion

The experiment was not designed for investigating the distribution of the labelled nitrogen between different parts of the system, but rather for studying to what extent different fungus species could influence the nitrogen uptake of pine seedlings. To obtain a criterion for this, only the shoot was analysed for nitrogen, the nitrogen content of this part of the plant being adjudged to be the most relevant, since the result of

root analyses may often be unreliable. It is impossible in practice to remove the sand and humus particles from roots without damaging them; of these, the former may considerably affect the results of the dry weight determination and hence the evaluation of the results. Where mycorrhiza-formation exists, it is, furthermore, not possible in an investigation of this kind to decide how great a part of the nitrogen content of the root system is derived from the mycelial mantle, and how much of the nitrogen in the mantle may be used by the plant. A series of investigations by Harley and his colleagues (reviewed by Harley, 1969) showed, however, that most of the phosphate absorbed by excised mycorrhizas of beech from a solution, could be recovered in the hyphal mantle of the mycorrhiza, which was accentuated the lower was the phosphate concentration of the solution. For a concentration of 0.32 mM, the hyphal mantle contained ca 90 per cent of the phosphate absorbed. An investigation by Carrodus (1967) showed that even ammonium nitrogen seemed to be primarily accumulated in the mantle.

The method used had both advantages and disadvantages. Of the advantages may be mentioned (1) that the shoot could be freely exposed and did not need to be surrounded by the sterile conditions affecting the root, (2) that the root system, despite the small volume available to it, could apparently develop normally—some plants, at the time when the experiment was terminated, had a main root almost 50 cm long—and (3) that it was possible to follow ocularly the development of the mycelium and the roots. Two of the disadvantages were important. Since the culturing time for the plants was nearly four months, it was necessary to supply them with water. This was done, as in the case of inoculation, by means of a syringe. In spite of rigorous precautions (the work was carried out in a sterile room with a sterile cannula and syringe, the lids of the capsules were wiped with 70 per cent ethanol, etc.), it was not possible to avoid contamination of the cultures, and as a result about half of the material had to be discarded. The second important disadvantage was that several slow-growing mycelia, such as isolates of *Amanita muscaria, Boletus subtomentosus* (C 118) and *Lactarius deliciosus,* seemed unable to tolerate the rough treatment involved in the preparation of the mycelium suspension. These species did not produce any mycelium after being inoculated into the capsules.

The results from the two experimental series (Exp. Nos. 1 and 2) concerning only cultures in which the fungi inoculated had developed well, as judged from the ocular assessment of the capsules, are reported in Tab. 6 & 7. For ease of evaluation of the results, these have also

been shown in the table (italics) as relative values. As the base for these, the values for cultures inoculated with *B. subtomentosus* (C 30) have been chosen, since four of the five uninoculated control cultures in one of the experimental series (No. 2) were spoiled by secondary infection. The values from the remaining plants were considered too poor a basis for assessment to be used. The various treatments will not be treated separately here, but by groups, and the distribution of the material between different groups has been based on the results obtained. Although relatively few cultures form the basis for the assessment it may, however, be proposed that the picture given below is in good agreement with that obtained in earlier experiments with the same isolates.

Group 1

Control, uninoculated—Tab. 6.

The needles of the plants were light green and exhibited clear symptoms of lack of nitrogen, as did also the well-developed root system (Fig. 9), which was provided with abundant short roots. A few of these were forked but were not swollen, as is the case in established mycorrhiza-formation.

The nitrogen which these plants had the opportunity of assimilating was both that occurring in mineralised form in the humus after gamma-sterilisation (ca 2.5 mg) and that supplied to the cultures in association with their inoculation (ca 2.0 mg), at the same time as the control cultures were given a corresponding volume of nutrient solution without mycelium. In addition, there was the 0.2—0.3 mg of nitrogen which is found in the seed (Räder-Roitzsch, 1957). In total, the nitrogen available to the plants should have been barely 5 mg, of which fully 50 μg was in the form of the isotope N^{15} in excess. Of this ca 50 per cent was recovered in the shoots of the plants in the control series.

Group 2

Inoculum: *Boletus subtomentosus* (C 30)—Tab. 6 & 7.

This isolate was quite exceptional, since it, the only one of those tested, resulted in vigorous plants (Fig. 9) with green to dark-green needles. The root system was well developed but the short roots were somewhat fewer in number compared with those of the control plants. As in the latter, some of the short roots were forked and were other-

Table 6. Exp. no. 1. Average shoot and root dry weight and amount of total N and atomic N^{15}-excess in shoots, etc., of over four-month-old seedlings of *Pinus silvestris* cultivated with the root system in plastic capsules filled with a gamma-sterilised (2.5 megarads) humus-sand-perlite mixture, inoculated with various mycorrhiza-forming and litter-decomposing fungi. The values are also given (italics) in relative terms with those for *Boletus subtomentosus* as basis. The experiment was performed in a phytotron with a photoperiod of 16 h, an illuminance of 30,000—40,000 lux and day and night temperatures of 20°C and 15°C, respectively.

As calculated per capsule, the humus mixture held initially ca 140 mg total N with ca 800 μg N^{15}-excess, of which 2.5 mg was exchangeable NH_4-N with 54 μg N^{15}-excess. In connection with inoculation the cultures were given 2.0 mg unlabelled NH_4-N. The seed contained 0.2—0.3 mg total N.

Inoculum	Number of cultures	Dry weight, mg			Needle colour	Presence of mycorrhiza	Analyses of the shoot			
							Total N		N^{15}-excess	
		shoot	root	total			%	mg	atom-%	μg
Boletus badius C 251	4	270 *48*	190 *68*	459 *54*	light green	none to moderate	0.87 *82*	2.4 ± 0.2 *41*	0.885 ± 0.010 *64*	21 ± 2 *26*
Boletus bovinus C 15	5	444 *78*	328 *118*	772 *91*	light green — green	scarce to abundant	0.81 *76*	3.5 ± 0.2 *59*	1.103 ± 0.013 *80*	38 ± 2 *46*
Boletus bovinus C 16	4	418 *74*	319 *114*	732 *87*	light green	scarce to moderate	0.70 *66*	3.0 ± 0.2 *51*	1.086 ± 0.027 *79*	32 ± 2 *39*
Boletus granulatus C 240	3	427 *75*	348 *125*	775 *92*	light green — green	scarce to moderate	0.93 *88*	4.0 ± 0.3 *58*	1.298 ± 0.024 *94*	52 ± 5 *63*
Boletus luteus C 243	5	417 *74*	329 *118*	746 *88*	light green — green	scarce to moderate	0.84 *79*	3.4 ± 0.2 *58*	1.129 ± 0.026 *82*	38 ± 1 *46*
Boletus subtomentosus C 30	5	566 *100*	279 *100*	845 *100*	green — dark green	none	1.06 *100*	5.9 ± 0.3 *100*	1.381 ± 0.017 *100*	82 ± 4 *100*

Table 6. ***(continued)***

Inoculum	Number of cultures	Dry weight, mg			Needle colour	Presence of mycorrhiza	Analyses of the shoot			
							Total N		N^{15}-excess	
		shoot	root	total			%	mg	atom-%	µg
Boletus variegatus C 35	4	435 *77*	377 *135*	812 *96*	light green — green	moderate to abundant	0.81 *76*	3.5±0.3 *59*	1.257±0.077 *91*	44±5 *54*
Hygrophoropsis aurantiaca C 135	3	307 *54*	274 *98*	581 *69*	light green	none	0.68 *64*	2.1±0.1 *36*	0.951±0.029 *69*	20±1 *24*
Paxillus involutus C 122	3	366 *65*	298 *107*	664 *79*	light green — green	none	0.75 *71*	2.8±0.5 *47*	1.210±0.019 *88*	34±6 *41*
Tricholoma nudum C 225	3	190 *34*	123 *44*	313 *37*	green — dark green	none	1.64 *155*	3.7±0.2 *63*	1.161±0.045 *84*	43±4 *52*
Control	3	393 *69*	268 *96*	661 *78*	light green	—	0.60 *57*	2.3±0.1 *39*	1.196±0.025 *87*	28±1 *34*

Table 7. Exp. no. 2. Average shoot and root dry weight, amount of total N and N^{15}-excess in shoots, etc., of over four-month-old seedlings of *Pinus silvestris*, and amount of exchangeable NH_4-N in the raw humus mixtures combined into a general sample at the end of the experiment. For further data, see Tab. 6.

Inoculum	Number of cultures	Dry weight, mg			Needle colour	Presence of mycorrhiza	Analyses of the shoot				Residual NH_4-N in raw humus mixture, mg
							Total N		N^{15}-excess		
		shoot	root	total			%	mg	atom-%	μg	
Boletus bovinus C 15	3	479 *73*	371 *99*	850 *82*	light green — green	scarce to abundant	0.77 *80*	3.5±0.1 *56*	0.985±0.029 *73*	35±2 *42*	0.6 *50*
Boletus granulatus C 240	4	587 *89*	416 *111*	1003 *97*	light green — green	none to moderate	0.70 *73*	4.1±0.1 *66*	1.276±0.012 *94*	52±2 *62*	0.7 *58*
Boletus subtomentosus C 30	3	656 *100*	376 *100*	1032 *100*	green — dark green	none	0.96 *100*	6.2±0.4 *100*	1.356±0.003 *100*	84±5 *100*	1.2 *100*
Boletus variegatus C 35	3	469 *71*	324 *86*	793 *77*	light green — green	scarce to moderate	0.81 *84*	3.7±0.2 *60*	1.183±0.005 *87*	44±3 *52*	0.7 *58*
Cenococcum graniforme D 10	3	168 *26*	168 *45*	336 *33*	light green	moderate	0.72 *75*	1.2±0.4 *19*	0.779±0.086 *57*	10±4 *12*	0.2 *17*
Paxillus involutus C 122	2	307 *47*	311 *83*	618 *60*	light green	none	0.69 *72*	2.1±0.2 *34*	1.121±0.147 *83*	24±5 *29*	0.5 *42*
Rhizopogon roseolus C 236	3	435 *66*	389 *103*	824 *80*	light green — green	scarce to abundant	0.73 *76*	3.2±0.2 *52*	1.119±0.026 *83*	35±2 *42*	0.5 *42*
Tricholoma nudum C 225	3	141 *21*	125 *33*	266 *26*	green — dark green	none	1.67 *174*	2.3±0.5 *37*	1.155±0.059 *85*	27±7 *32*	4.4 *367*

Table 7. ***(continued)***

Inoculum	Number of cultures	Dry weight, mg			Needle colour	Presence of mycorrhiza	Analyses of the shoot				Residual NH_4-N in raw humus mixture, mg
							Total N		N^{15}-excess		
		shoot	root	total			%	mg	atom-%	μg	
B. bovinus + *B. subtomentosus*	3	664 *101*	366 *97*	1030 *100*	green — dark green	none	0.98 *102*	6.5±0.3 *105*	1.324±0.031 *98*	86±2 *102*	1.2 *100*
B. granulatus + *B. subtomentosus*	3	588 *90*	379 *101*	967 *94*	dark green	none	1.12 *117*	6.6±<0.1 *106*	1.422±0.006 *105*	93±1 *111*	1.3 *108*
C. graniforme + *B. subtomentosus*	3	535 *82*	312 *83*	847 *82*	light green — green	none	1.02 *106*	5.3±<0.1 *85*	1.436±0.047 *106*	77±1 *92*	1.0 *83*
T. nudum + *B. subtomentosus*	4	574 *88*	392 *104*	966 *94*	light green — dark green	none	1.08 *113*	5.8±0.2 *94*	1.385±0.025 *102*	81±2 *96*	1.2 *100*
B. variegatus + *T. nudum*	1*	275 *42*	550 *146*	825 *80*	green	scarce	1.08 *113*	3.0 *48*	1.168 *86*	35 *42*	0.9 *75*
	3**	217 *33*	151 *40*	368 *36*	dark green	none	1.69 *176*	3.6±0.5 *58*	1.242±0.024 *92*	45±7 *53*	3.5 *292*
B. variegatus + *Rh. roseolus*	4	390 *59*	419 *111*	809 *78*	light green — green	scarce to moderate	0.78 *81*	3.0±0.2 *48*	1.158±0.010 *85*	35±2 *42*	0.7 *58*

* Culture mainly influenced by *B. variegatus*
** Cultures mainly influenced by *T. nudum*

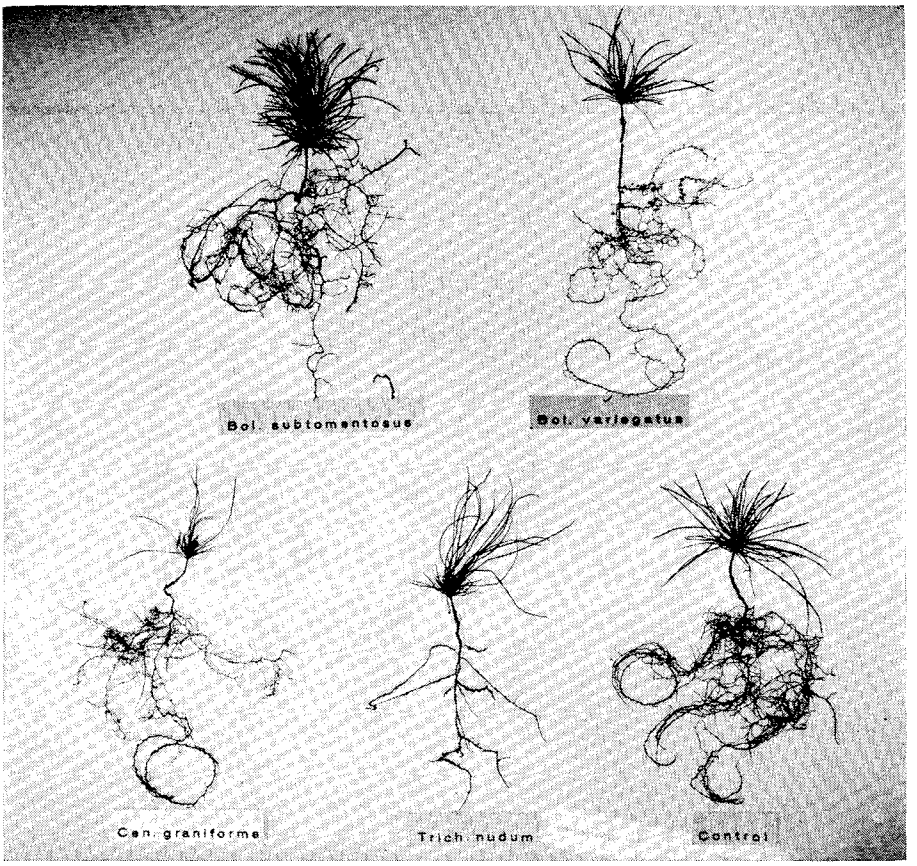

Fig. 9. Over four-month-old seedlings of *Pinus silvestris* from capsule cultures inoculated with mycelium suspensions of different soil fungi.

wise of similar appearance. In spite of the abundant occurrence on the roots of superficial white mycelium, no mycorrhiza-formation was found, which is in agreement with previous experience (Tab. 3).

The nitrogen analyses showed that the shoots alone contained ca 6 mg of nitrogen, of which fully 80 μg N^{15} in excess, or in other words a larger quantity than was to be found in a form available to the plants in the capsules, *even though the mycelium had also competed with the plants for that nitrogen.* This indicates that the inoculation had brought about a net mineralisation of nitrogen, i.e. a surplus of nitrogen in mineralised form which the mycelium itself did not consume. Together with the circumstance that the mycelium, in spite of its good growth, did not form mycorrhiza, this result reinforces the view previously

advanced, that *this isolate* of *B. subtomentosus* is to be considered as a litter-decomposer, having the ability actively to liberate organically bound nitrogen from the raw humus.

Group 3

Inoculum: *Boletus granulatus* (C 240)—Tab. 6 & 7.
B. variegatus (C 35)—Tab. 6 & 7.
B. luteus (C 243)—Tab. 6.
B. bovinus (C 15)—Tab. 6 & 7.
Rhizopogon roseolus (C 236)—Tab. 7.
B. bovinus (C 16)—Tab. 6.
B. badius (C 251)—Tab. 6.

The fungi assigned to this group had in common, amongst other things, the fact that they had formed mycorrhiza with the pine seedlings. In spite of the apparently equally good mycelium development in the capsules, the frequency of mycorrhiza varied considerably within one and the same experimental series. The mycorrhiza were predominantly of A-type (according to Björkman, 1942), being unbranched or forked (Fig. 10). *B. luteus* and *B. variegatus* gave rise, however, mainly to mycorrhiza of B-type. The mycorrhizas were white in colour or in the case of *Rh. roseolus,* white with a slight tinge of brown. The fungal sheet was relatively compact and 10—20 μ thick in mycorrhiza with *B. badius, B. granulatus* (Fig. 11), *B. luteus* and *Rh. roseolus,* while it was somewhat more loose and 25—40 μ thick with *B. bovinus* (Fig. 11) and *B. variegatus.* The needles showed in most cases symptoms of nitrogen deficiency. The shoot dry weight varied, but was on the average 30 per cent lower than that in plants inoculated with *B. subtomentosus* (C 30). The root dry weight, on the same basis of comparison, was generally slightly higher, which seems at least partly to have been caused by the soil particles' having adhered to the roots and not having been removed, even though the roots were rinsed in running water.

The inoculation of the capsules with the fungi in this group, *B. badius* excepted, showed a tendency to cause a net mineralisation of the bound nitrogen in the humus, since the amount of total N and N^{15} in excess in the shoots was greater than that in the control seedlings. (The assessment of the result for *Rh. roseolus* can only be an estimate, since a basis for comparison was lacking). This tendency was greatest in *B. granulatus* and *B. variegatus,* but decreased in the order in which the fungi are listed in the introduction. The variation within the various

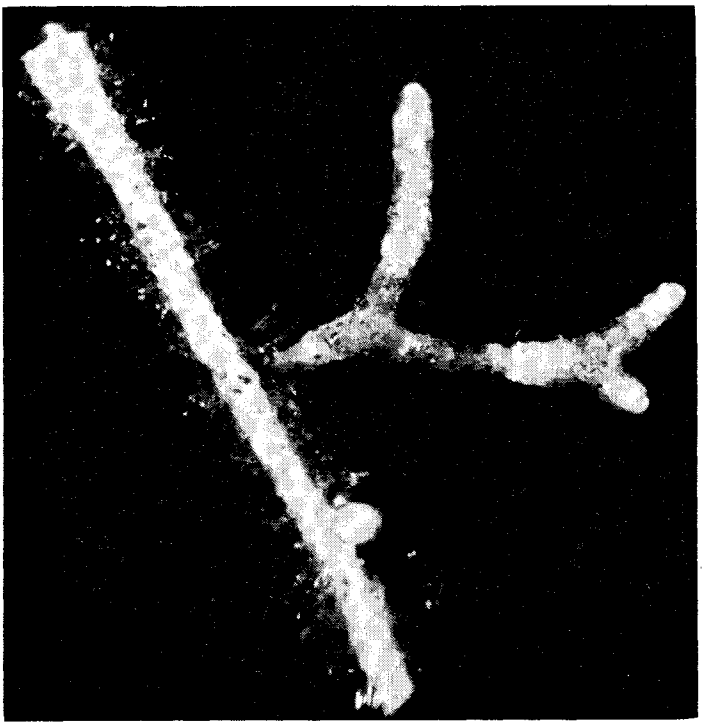

Fig. 10. Mycorrhiza of A-type (according to Björkman's (1942) classification scheme) in *Pinus silvestris* in capsule cultures inoculated with *Boletus bovinus*, × 7.

experimental series was, however, so great that only these two species can be considered on good grounds to have given rise to the conclusion.

This result somewhat contradicts that in the preceding experimental series (Cap. V), since the mycorrhiza-forming isolates in that series showed no tendency to be able to utilise the organically bound humus nitrogen. The experimental conditions were, however, in many respects so dissimilar that several factors could have caused this difference. The possibility that the plant itself may have influenced the function of the fungi in this respect cannot be ignored, and intra-cellular enzymes may also have played some part. In the previous experimental series, pieces of agar containing mycelium were used as inoculum, and the experiments lasted only three weeks. With this inoculation procedure and during this short space of time, it is scarcely likely that autolysis of the mycelium can have occurred. In the present experimental series, however, a mycelium suspension was used as inoculum and the experiments lasted fully five months from the first inoculation.

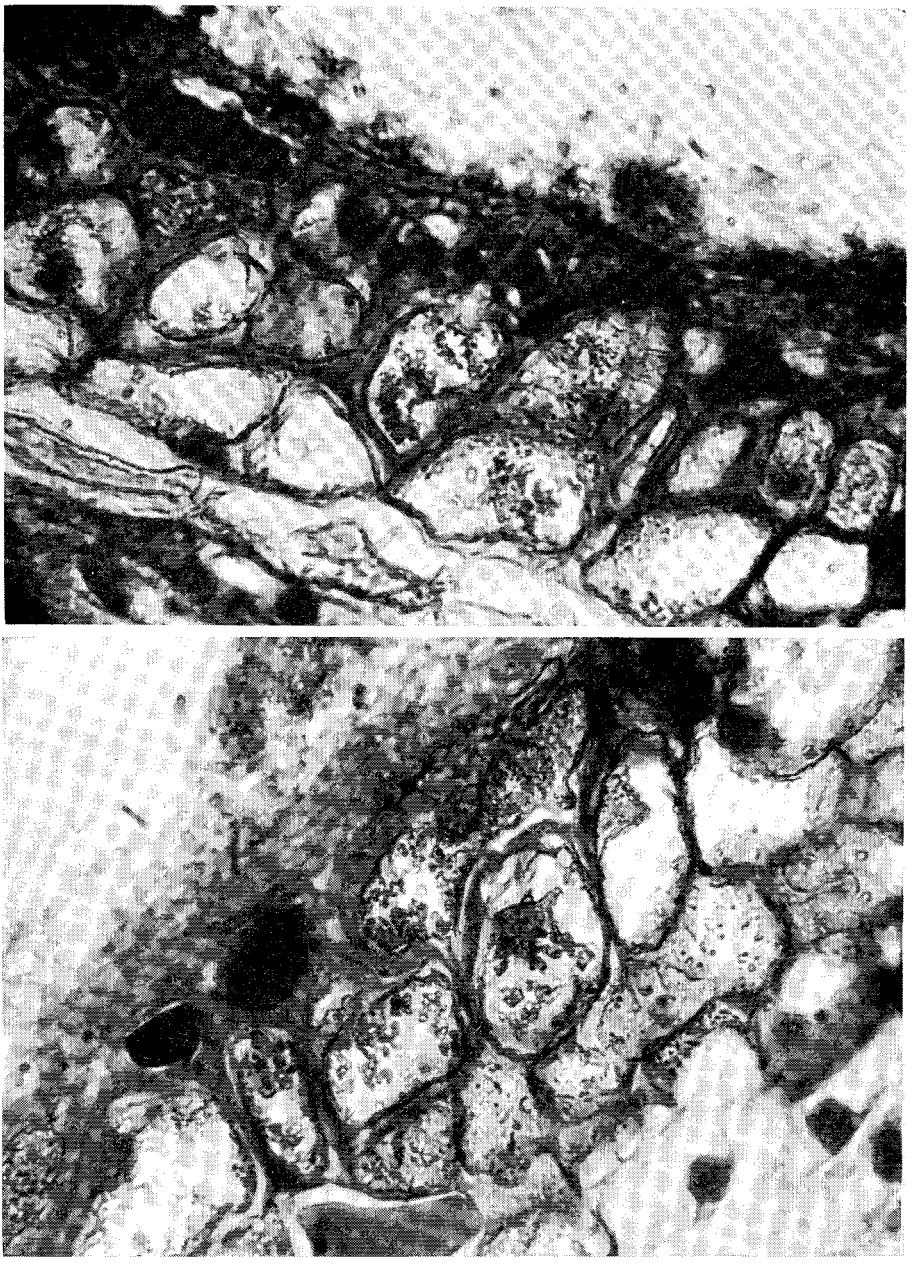

Fig. 11. Longitudinal section of an ectotrophic mycorrhiza in *Pinus silvestris*, formed in a capsule culture inoculated with a mycelium suspension of *Boletus granulatus* (above) and *B. bovinus* (below), × 400.

Both the production of the suspension and the comparatively long experimental period may have resulted in the partial death and autolysis of the mycelium with consequent liberation of intra-cellular enzymes. This might be the cause of the net mineralisation which occurred, since it seems not improbable that this enzyme complex, apart from causing the break-down of the various components of the dead mycelium, may also have affected the surrounding medium.

As was mentioned above, the frequency of mycorrhiza varied considerably within the experimental series. This made it possible to make a comparison between plants having the highest and the lowest frequency, respectively (Tab. 8). An analysis of the individual results thus showed that the shoot dry weight was in general lower in plants with the highest frequency of mycorrhiza or, on the average, 20 per cent. This was most marked in the cultures inoculated with *Boletus bovinus* (C 15). In these, as in those inoculated with *Rhizopogon roseolus,* the variation in the occurrence of mycorrhiza was largest. The same tendency existed even for the amount of total nitrogen in the shoots. The cause of this situation is probably to be found in the storage function which the mycorrhiza obviously possess. Thus Kramer & Wilbur (1949) showed that phosphate accumulated to a considerably greater extent in mycorrhizas than in uninfected short roots, and subsequent investigations have demonstrated that the hyphal mantle is the part of the mycorrhiza responsible for this accumulation (Harley & McCready, 1952a, 1952b; Clode, 1956). This also applies to ammonium nitrogen (Carrodus, 1966, 1967). As regards the transport of phosphate to the shoots, Morrison (1957) found that this was greater during the first two weeks in pine seedlings lacking mycorrhiza, but that the situation was later reversed. Ritter (1965) investigated the transport of radioactive phosphate from the fruit-bodies of *Hebeloma mesophaeum* to pine seedlings in pot cultures, and obtained during the first five days only a slight increase of the P^{32}-content of the needles, while it became considerably greater during the following five days; this depended, in Ritter's opinion, on the mycorrhiza's full storage capacity having then been attained. It seems not improbable that the amount of nitrogen in assimilable form in the humus mixture used in the present investigation was so slight that the full storage capacity was never attained during the period of the experiment. By analogy with the result of Ritter's investigation, this should imply a reduced translocation of nitrogen to the shoots, and be most noticeable in plants with the highest frequency of mycorrhiza, which also tended to be the case in the present investigation.

Table 8. Average shoot dry weight and amount of total N in the shoot of seedlings with smallest and greatest mycorrhiza frequency, respectively. The values are derived from Exp. nos. 1 and 2 (Tab. 6 and 7).

Inoculum	Exp. no.	Presence of mycorrhiza	Number of cultures	Shoot dry weight		Total N in shoot	
				mg.	rel.	mg.	rel.
Boletus badius	1	none	2	295	100	2.7	100
C 251		moderate	1	240	81	2.2	81
Boletus bovinus	1	scarce	2	524	100	3.7	100
C 15		abundant	1	270	52	2.8	76
	2	scarce	2	561	100	3.6	100
		abundant	1	316	56	3.3	92
Boletus bovinus	1	scarce	3	428	100	3.0	100
C 16		moderate	1	391	91	2.7	90
Boletus granulatus	1	scarce	2	457	100	4.3	100
C 240		moderate	1	369	81	3.4	79
	2	none	1	660	100	4.2	100
		moderate	1	503	76	3.7	88
Boletus luteus	1	scarce	2	416	100	3.3	100
C 243		moderate	3	417	100	3.5	106
Boletus variegatus	1	moderate	2	412	100	3.5	100
C 35		abundant	2	458	111	3.5	100
	2	scarce	1	512	100	4.0	100
		moderate	2	385	75	3.3	83
Rhizopogon roseolus	2	scarce	2	474	100	3.4	100
C 236		abundant	1	375	79	2.7	79
Mean value		smallest	19	474	100	3.6	100
		greatest	14	372	80	3.1	87

Group 4

Inoculum: *Hygrophoropsis aurantiaca* (C 135)—Tab. 6.
Paxillus involutus (C 122)—Tab. 6 & 7.

The fungi in this group were characterised by their not forming mycorrhiza with the pine seedlings. Where *H. aurantiaca* is concerned, this was not unexpected, since it is considered to be a litter-decomposer, as is indicated by the enzyme investigation previously reported. The ecological status of *P. involutus* is uncertain. Observations of its occurrence in nature indicate that this species may be a mycorrhiza-former with a number of different tree species, both conifers and broad-leaved trees (Trappe, 1962, p. 564) which, however, is contradicted by the results of Romell's (1939) investigation, in that he

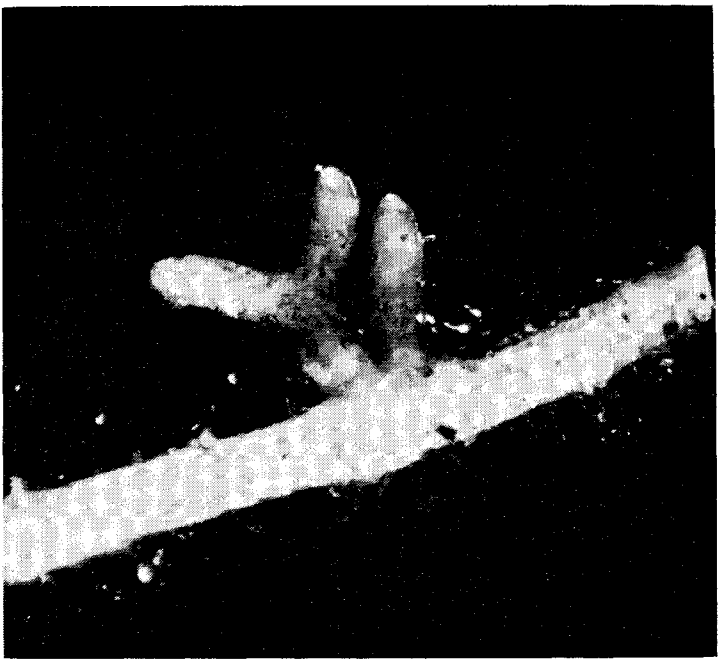

Fig. 12. Mycorrhiza-like short roots in *Pinus silvestris* in capsule culture inoculated with a mycelium suspension of *Hygrophoropsis aurantiaca*, × 10.

obtained sporophore formation by this species in the root-isolated sample plots he laid out in the forest. Neither has the formation of mycorrhiza so far been reported from pure culture synthesis experiments.

As regards shoot and root development in the seedlings of this group, this was similar to that in the controls, except that more short roots were forked. These also differed in morphological respects since in the inoculated cultures they were swollen and reminiscent of true mycorrhizas (Fig. 12). Microscopic study showed, however, that no mycorrhiza-formation existed (Fig. 13). Such an influence on the part of the fungus on the morphology of pine roots has been noted earlier, with both mycorrhiza-forming and litter-decomposing species (Slankis, 1948; Levisohn, 1952; Turner, 1962).

The values from the nitrogen analyses were of the same order as those in the control plants. This indicates that the two isolates in this group had not the ability to bring about a net mineralisation, which agrees with the results of the previous experimental series.

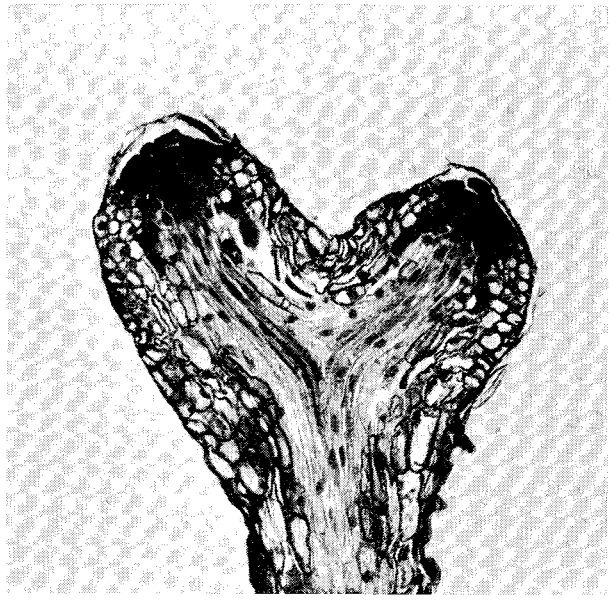

Fig. 13. Longitudinal section of a forked, non-mycorrhizal short root in *Pinus silvestris* in capsule culture inoculated with a mycelium suspension of *Paxillus involutus*, × 40.

Group 5

Inoculum: *Cenococcum graniforme* (D 10)—Tab. 7.
Tricholoma nudum (C 225)—Tab. 6 & 7.

Since both of these isolates had a clearly deleterious effect on plant development (Fig. 9), they have been united into this group. It is, however, quite clear that they have affected the plants in different ways. Thus those inoculated with *C. graniforme* had light green needles and a morphologically normal root system with a moderate occurrence of Dn-type mycorrhiza. The needles of plants inoculated with *T. nudum* were, in contrast, quite clearly dark green and the root system was misshapen, since it lacked practically all short roots. No trace could be found of mycelium on the roots, although good mycelium development had taken place in the capsules.

The nitrogen analyses for the plants inoculated with *C. graniforme* showed that the shoots contained small amounts of nitrogen, the lowest measured, which indicates that inoculation with this species had caused a net immobilisation of the assimilable nitrogen in the humus mixture, i.e. immobilisation exceeded mineralisation. The values

for the plants inoculated with *T. nudum* are left as an open question, being totally irrelevant, since the vital functions of the plants had clearly been injured by the action of the fungus. They will, however, be commented upon at a later stage.

Group 6 (Tab. 7)

Inoculum: *B. subtomentosus* (C 30)	+*B. bovinus* (C 15)
„	+*B. granulatus* (C 240)
„	+*C. graniforme* (D 10)
„	+*T. nudum* (C 225)
B. variegatus (C 35)	+ „
„	+*Rh. roseolus* (C 236)

The aim of this part of the experiment was to investigate whether there was "teamwork" between fungi, to the advantage of the plants' nitrogen supply. In the first place, the combination of *B. subtomentosus* (C 30) with a true mycorrhiza-former seemed to be of most interest, since there was a possibility that the former might be responsible for the mineralisation of the bound nitrogen and the latter for the transfer of the released nitrogen to the plants. However, the conditions necessary for permitting assessment of this were not fulfilled, since mycorrhiza never became established in any combination which included *B. subtomentosus,* and in all treatments with this fungus, analysis values of the same order of magnitude as those for the fungus in single culture were obtained.

The inhibition of mycorrhiza-formation may have been caused by several factors. One explanation might be that *B. subtomentosus* suppressed the fungus inoculated together with it, by producing toxic substances. Joint culture on malt agar dishes indicated, however, that this was not the case. The failure of mycorrhiza-formation could also be explained by Björkman's (1942) carbohydrate theory, since it is possible that *B. subtomentosus* released so much nitrogen that plants with access to it used up the excess of soluble carbohydrates in their roots and consequently became unattractive to the mycorrhiza-forming mycelium. It seems, however, more probable that at an early stage the rapidly growing mycelium of *B. subtomentosus* competed out the mycelium inoculated together with it, by itself taking possession of the glucose available to the fungi in the mycelium suspension in the capsules. In support of this explanation is the fact that no trace could be found of the black mycelium formed by *C. graniforme* in the

Fig. 14. Cultures of over four-month-old seedlings of *Pinus silvestris* inoculated with a mycelium suspension of *Boletus subtomentosus* and *Tricholoma nudum*, both individually and jointly.

capsules in which this fungus accompanied *B. subtomentosus,* but only of white mycelium. This could also explain why the deleterious influence exercised on the seedlings by *T. nudum* in single culture failed to appear (Fig. 14).

In the capsules inoculated with *B. variegatus* and *T. nudum* an "either-or" situation existed. As may be seen from Tab. 7, the former fungus was dominant in one of the four capsules. The latter dominated in the remaining three, but there was a tendency for the presence of *B. variegatus* to bring about some reduction of the negative influence which *T. nudum* had on seedlings in single culture. Finally, as regards the combination of *B. variegatus* and *Rh. roseolus,* results were obtained on a level with those for the fungi in single culture. Thus no combination effect was to be observed. Since the mycorrhiza were mainly white with a light-brown tinge, this indicated that *Rh. roseolus* was the dominant of the two.

In the treatment reported in Tab. 7, the humus mixture was investigated at the end of the experiment for its content of exchangeable ammonium nitrogen. For this purpose the humus mixtures from the capsules in each treatment were combined into a general sample.

The analysis values obtained are shown in the table, and reinforce earlier conclusions. Thus the capsules inoculated with *B. subtomentosus* as a single culture or a combined culture contained, on the average, twice as much exchangeable ammonium nitrogen as those inoculated with the fungi in group 3 *(B. bovinus, B. granulatus, B. variegatus* and *Rhizopogon roseolus)* and group 4 *(Paxillus involutus)*, even though the shoots contained a considerably larger amount of total nitrogen. This clearly indicates that inoculation of *B. subtomentosus* brought about a greater net mineralisation than that caused by the inoculation of the above-named fungi. Furthermore, the low value for capsules inoculated with *Cenococcum graniforme* (group 5) indicates that this isolate had caused a net immobilisation. Of interest, too, were the high values obtained for cultures inoculated with *T. nudum*, either in single culture or where it had dominated in the combinations with *B. variegatus* (according to the response to inoculation expressed in plant development). This was not in itself surprising, since the shoots contained small amounts of total nitrogen. If, however, both nitrogen items are summed, values of the same order as those from cultures inoculated with *B. subtomentosus* are obtained; in other words, higher than those in cultures inoculated with the fungi in groups 3 and 4. This indicates that the inoculation of *T. nudum* brought about a net mineralisation of humus nitrogen, which was not unexpected, considering the response the isolate gave in the enzyme investigation.

By way of summary, it may be stated that under the experimental conditions:

(1) Inoculation with the mycorrhiza-forming isolates of *Boletus granulatus* (C 240) and *B. variegatus* (C 35) brought about a net mineralisation of nitrogen, which was, however, of comparatively small extent. A tendency towards this was obtained with a few other isolates. Mineralisation may, however, have been caused by intra-cellular enzymes. Mycorrhiza-formation seems primarily to result more in a decrease than in an increase of the dry weight and amount of nitrogen in the shoots.

(2) Inoculation with the isolate of *Cenococcum graniforme* caused a marked net immobilisation and hence a nitrogen depression in the plants.

(3) Inoculation with the three litter-decomposing isolates gave completely divergent responses in the plants

a. *Boletus subtomentosus* (C 30) gave rise to a comparatively high net mineralisation, which considerably stimulated the growth of the plants.

b. *Hygrophoropsis aurantiaca* (C 135) resulted in a *status quo.*
c. *Tricholoma nudum* (C 225) gave rise to a comparatively high net mineralisation, but had a deleterious influence on the growth of the plants.

Chapter VII. Conclusions and general discussion

In the first two experimental series reported in the present paper, the culturing conditions for the fungi were completely artificial, while in the two subsequent series, an attempt was made to imitate to some extent the conditions existing in nature. In spite of this, several factors were naturally totally artificial, and one of these was the absence of the entire soil microflora and with it, of the interplay, the competition and the mutual influence which exists between microorganisms under natural conditions. It is, therefore, not possible to generalise from the results obtained to the actual conditions, but they can give indications and provide some basis for the interpretation of information from other investigations.

The main aim of the present investigation was, as is evident, to attempt to elucidate whether ectotropic mycorrhiza-formers are able to release actively to any extent the organically bound nitrogen existing in the raw humus in a form which cannot be assimilated by trees, and thereby be of advantage to their partner in respect of its nitrogen supply. Although no completely unequivocal answer could be obtained to this question, the investigation gave a clear indication that the mycorrhiza-forming species studied either could not do so, or could do so to such a limited extent as to have no real importance. This conclusion is based on the results from two of the experimental series, in which the question posed in the one was to be elucidated by an indirect, and in the other by a direct, procedure. Thus the enzyme investigation showed that mycorrhiza-formers, in contrast to litter-decomposers, produced in general small amounts of extra-cellular enzymes of the kind considered to give rise to the break-down of organic substances, which is in good agreement with earlier experience (Lyr, 1963a; Ritter, 1964). This circumstance can in itself not be taken as a proof of their incapacity to mineralise bound humus nitrogen, but indicates that they are in character a group of inactive organisms. Neither did the experiment with glucose/humus agar dishes, which included a larger number of isolates of mycorrhiza-formers than was investigated in respect of enzyme production, give any positive indication; this was, however, the case with three of the litter-decomposing isolates. In the subsequent capsule experiment, some net mineralisa-

tion was certainly obtained with a few of the mycorrhiza-forming isolates, but since there was a possibility that this was caused by intra-cellular enzymes liberated by autolysis, the result cannot be taken as indicating that the isolate in question had actively brought about the mineralisation which occurred.

A shortcoming inherent in the present work, as in many other mycorrhiza investigations which include inoculation with pure cultures, is that, for instance, the genera *Lactarius*, *Russula* and *Cantharellus* are seldom or never represented. This is explained by the fact that it is particularly difficult to produce pure cultures of species belonging to these genera, since the fruit-bodies are often intimately associated with bacteria and in successful cases the isolates are generally so slow-growing on normal culture media that it is not practically possible to work with them. These three genera are, however, considered to include ectotrophic mycorrhiza-formers, for which reason the possibility cannot be ignored that species other than those tested in the present investigation are able to mineralise the humus nitrogen; in this context it may be pointed out that the only representative for the three above-mentioned genera viz., *Lactarius deliciosus*, gave both in the enzyme investigation and in the experiment with the glucose/humus agar dishes a slight positive indication in this respect.

The results for the two isolates of *Boletus subtomentosus* aroused interest, since they so obviously diverged. The one isolate (C 118) exhibited a pattern of behaviour which fully agrees with that of other mycorrhiza-formers, since it was comparatively slow-growing and had a low enzyme activity. In synthesis experiments it also formed mycorrhiza with various species of pine. The other isolate (C 30), by way of contrast, was fast-growing and showed a high enzyme activity, on a level with that of the litter-decomposers. Although this isolate has been used for inoculation in a number of different experimental series in which the experimental conditions have varied considerably, no mycorrhiza-formation has ever been discovered; Björkman (1970) also had the same experience in his pot culture experiments in growth chambers. Furthermore, the isolates differed in respect of their capacity to make use of different nitrogen sources. Thus C 118 could make use of neither ammonium nor nitrate, and of the organic nitrogen sources on which it was tested, it could assimilate only L-asparagine. In contrast, C 30 could make use of several different nitrogen sources, both inorganic and organic. These results indicate that there exist two physiologically and ecologically widely separated races of the same species, the one unequivocally resembling the mycorrhiza-formers

and the other reminiscent of the litter-decomposers. Isolates which exhibit good affinity with these two races have been met with earlier. Thus Modess (1941) obtained mycorrhiza-formation with a slow-growing mycelium of *B. subtomentosus*, while the isolate included in Lindeberg's (1948) experiment "behaved as a litter-decomposing fungus". This was fast-growing and gave rise to a decrease of 49 per cent in the dry weight of a substrate consisting of straw and leaves after about six months' incubation. Romell (1939) found fruit-bodies of this species on experimental plots completely isolated from living tree roots. On the basis of this he considered that *B. subtomentosus* was to be regarded as a facultative mycorrhiza-former or in other words a species which usually forms mycorrhiza but which if compelled to do so, can also behave as a saprophyte. That there exists such a transition between symbiotic and saprophytic fungi is apparent, and Norkrans (1950) has demonstrated this to be the case for an isolate of *Tricholoma fumosum*. The same isolate both formed mycorrhiza and possessed the ability to produce cellulase. It seems not improbable that the situation might be similar for the isolates of *Clitopilus prunulus* and *T. albobrunneum* in the present investigation; these formed cellulase+ proteinase and pectinase, respectively, even if the first-named isolate failed to form mycorrhiza in an experiment under controlled conditions (Lundeberg, 1967). The isolate of *T. albobrunneum* has not been tested in respect of mycorrhiza-forming ability. The marked unwillingness to form mycorrhiza exhibited by *B. subtomentosus* (C 30), makes it likely that this isolate stands closer to the saprophytic fungi than to the facultative mycorrhiza-formers, which underlines the view previously expressed by other authors, namely that there is a continuous transition between one ecological group and another.

In this context Levisohn's investigation (1956) should be mentioned. She found that "free-living mycorrhizal mycelia" of *Rhizopogon luteolus* but above all, of *Boletus scaber* clearly stimulated the development of tree seedlings without the establishment of mycorrhiza. Levisohn put forward the view that the stimulation may have been caused by the fungi having broken down the soil organic matter and thus released nutrient to the benefit of the plants. There seems, therefore, to be a clear parallel between Levisohn's isolate and *B. subtomentosus* (C 30) since this, too, had a clearly stimulating influence on plant growth (see also Björkman, 1970). Since *B. subtomentosus* (C 30) could in fact break down soil organic matter it seems probable that this was also the case with Levisohn's isolates.

Even though many common mycorrhiza-forming fungi seem to lack

the ability to take an active part in the mineralisation of the organically bound nitrogen, it seems probable that some of them are better adapted than others to aid the higher plants with their nutrient supply. Ritter & Lyr (1965) have demonstrated that this is the case with phosphate supply. They found that the uptake of phosphate in pine plants inoculated with *Boletus (Suillus) luteus* was considerably greater than that in plants inoculated with *Amanita muscaria*. The experimental series reported in Cap. IV above indicate that there were large differences between different isolates in respect of the ability to utilise various nitrogen sources. Thus, for instance, the isolate of *A. rubescens* could utilise only ammonium, and *B. subtomentosus* (C 118) only L-asparagine, of the nitrogen sources included in the experiment. In contrast to this, however, the isolates of *B. luteus*, *Rhizopogon luteolus* and *Tricholoma pessundatum* exhibited a considerably wider spectrum in that they could make use of both ammonium and nitrate as well as of several simple organic nitrogen sources, and it seems likely that they would have a greater possibility of supporting their partner in its attempts to satisfy its nitrogen requirements.

By using the method developed by Moser (1963), it is possible to introduce a desired mycorrhiza-forming mycelium into a nursery, so that the plants at the time of planting in forest soil possess a suitable partner. It would also be of value to equip the plants with a companion such as *B. subtomentosus* (C 30), which is able to mineralise humus nitrogen and also to stimulate plant growth (cf. *T. nudum*, Cap. VI). Moser's method is, however, probably not applicable, since at least this isolate seems unwilling to form mycorrhiza. There exists, therefore, no guarantee that it will accompany the plants when they leave the nursery. In recent years a method differing from the conventional method has come increasingly into use. This is based on the plants' being grown on a substrate in pots of compressed peat. This takes place in the greenhouse; when the plants have reached the desired size and age the whole unit is planted out either manually or mechanically (Froland, 1968; Sirén, 1968). In the application of this method it might be possible to inoculate the substrate with a mycelium suspension and in this way to guarantee that the plants are provided with a serviceable mycelium at the time of planting of the unit.

Summary

The investigation was carried out with a number of isolates of mycorrhiza-formers, belonging mainly to the genera *Amanita, Boletus, Rhizopogon* and *Tricholoma*. For comparison of two groups of ecologically dissimilar soil fungi, isolates of litter-decomposers were also included.

To establish the grouping of the isolates in respect of ecology, 23 of them were investigated for their ability to produce the extra-cellular enzymes cellulase, pectinase, proteinase and laccase. The supposed mycorrhiza-formers were generally clearly inferior to the presumed litter-decomposers in this respect. Some deviations were, however, noted, since the isolates of *Clitopilus prunulus, Lactarius deliciosus* and *Tricholoma albobrunneum* formed cellulase and proteinase, laccase and pectinase, respectively, to a relatively large extent. Amongst isolates of *Boletus subtomentosus,* two races were distinguishable, one mycorrhiza-forming, with a low enzyme activity and one like the litter-decomposers in its behaviour. *Tricholoma nudum*—supposedly a litter-decomposer—exhibited remarkably low cellulase and pectinase activity.

Ammonium nitrogen and L-asparagine were the nitrogen sources most readily utilised, but others acceptable to several species were glycine, acetamide and nitrate nitrogen. Only two out of 28 species, viz. *Boletus elegans* and *Tricholoma pessundatum,* were capable of utilising D-glucosamine. In contrast diethylamine, L-proline and pyridine were almost unusable as nitrogen sources, as also was nitrite, which had throughout a toxic effect on the fungi. No divergence could be traced between mycorrhiza-formers and litter-decomposers, other than a tendency for the former to have a greater possibility of utilising nitrate nitrogen and the latter amide nitrogen. Some isolates could make use only of a single nitrogen source, organic or inorganic, while others had a considerably wider spectrum; it is supposed that mycorrhiza-formers of the latter type have a greater likelihood of being able to assist the higher symbiont in satisfying its nitrogen requirements.

The ability to utilise organically bound soil nitrogen was investigated in specially prepared plastic petri dishes. Gamma-sterilised, N^{15}-labelled raw humus constituted the nitrogen source; the result indicated that the mycorrhiza-formers included in the experiment and fully half of the litter-decomposers, could not do so under the experimental conditions. A positive indication in this respect was obtained only for

Agaricus campestris, Fomes annosus and an isolate of *Boletus subtomentosus*, which in the enzyme investigation exhibited affinity with the litter-decomposers.

In the joint culture of pine seedlings on a gamma-sterilised mixture of N^{15}-labelled raw humus, sand and perlite, a few isolates of mycorrhiza-forming *Boletus* species gave rise to a net mineralisation of the bound humus nitrogen which, however, did not notably affect the development of the seedlings. Mycorrhiza-formation seems in itself to have inhibited rather than stimulated plant development under the experimental conditions. The reason for this is discussed. The mycorrhiza-forming fungus *Cenococcum graniforme* caused a clear net immobilisation of the exchangeable nitrogen, which brought about marked nitrogen depression in the seedlings. Inoculation with an isolate of *Boletus subtomentosus*, the same as that used in the previous experimental series, caused a relatively high net mineralisation which considerably stimulated the growth of the seedlings. *Tricholoma nudum* also gave rise to a net mineralisation of nitrogen, but had in spite of this a clearly deleterious effect on plant growth.

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Sammanfattning

Undersökningen utfördes med ett flertal isolat av mykorrhizabildare huvudsakligen tillhörande släktena *Amanita*, *Boletus*, *Rhizopogon* och *Tricholoma*. För att möjliggöra jämförelse mellan ett par i ekologiskt avseende olika grupper av marksvampar, voro även isolat av förnanedbrytare inkluderade i undersökningen.

Med avsikt att söka säkerställa isolatens gruppering i ekologiskt avseende undersöktes tjugotre av dem på förmåga att producera de extracellulära enzymerna cellulas, pektinas, proteinas och laccas. Det befanns att de förmodade mykorrhizabildarna i allmänhet voro klart underlägsna de presumtiva förnanedbrytarna i detta avseende. En del avvikelser kunde dock noteras, då isolaten av *Clitopilus prunulus*, *Lactarius deliciosus* och *Tricholoma alboBrunneum* bildade cellulas och proteinas, laccas respektive pektinas av förhållandevis hög omfattning. Bland isolat av *Boletus subtomentosus* kunde två raser urskiljas, en mykorrhizabildande med låg enzymaktivitet och en i sitt agerande lik förnanedbrytare. *Tricholoma nudum* — en förmodad förnanedbrytare — visade anmärkningsvärd låg cellulas- och pektinasaktivitet.

Ammonium och L-asparagin voro de kvävekällor, som bäst kunde utnyttjas, men acceptabla sådana voro för flera arter även glycin, acetamid och nitratkväve. Enbart två av tjugoåtta arter, nämligen *Boletus elegans* och *Tricholoma pessundatum*, voro kapabla att utnyttja D-glykosamin. Däremot voro dietylamin, L-prolin och pyridin i det närmaste obrukbara som kvävekällor liksom nitrit, vilket genomgående hade en toxisk effekt på svamparna. Någon divergens mellan mykorrhizabildare och förnanedbrytare kunde ej spåras förutom en tendens till att de förra hade större möjlighet att utnyttja nitratkväve och de senare amidkväve. En del isolat kunde blott tillgodogöra sig någon enstaka kvävekälla, organisk eller oorganisk, medan andra uppvisade ett betydligt bredare spektrum och det förmodas att mykorrhizabildare av det senare slaget ha större möjlighet att stödja den högre symbionten i dess kväveförsörjning.

Förmågan att utnyttja organiskt bundet markkväve undersöktes i specialpreparerade plastpetriskålar. Gammasteriliserad, N^{15}-märkt råhumus utgjorde därvid kvävekälla och resultatet indikerade att de i försöket ingående mykorrhizabildarna liksom drygt hälften av förnanedbrytarna ej hade möjlighet därtill under rådande försöksbetingelser. En positiv indikation i detta hänseende erhölls enbart för *Agaricus campestris*, *Fomes annosus* samt ett isolat av *Boletus subtomentosus*, som i enzymundersökningen visade samhörighet med förnanedbrytare.

Vid samkultivering med tallplantor i en gammasteriliserad blandning av N^{15}-märkt råhumus, sand och perlit gav ett par isolat av mykorrhizabildande *Boletus*-arter upphov till en nettomineralisering av det bundna humuskvävet, vilket dock ej nämnvärt påverkade plantornas utveckling. Mykorrhizabildningen i sig själv snarast hämmade än stimulerade plantutvecklingen under de

försöksbetingelser, som rådde, och orsaken till detta har sökt tolkas. Den mykorrhizabildande svampen *Cenococcum graniforme* orsakade en påtaglig nettoimmobilisering av för plantorna tillgängligt kväve, vilket medförde en markant kvävedepression hos plantorna. Ympningen med ett isolat av *Boletus subtomentosus*, samma som det i föregående försöksserie, medförde en förhållandevis hög nettomineralisering, vilket påtagligt stimulerade plantornas tillväxt. Även *Tricholoma nudum* gav upphov till en nettomineralisering av kväve, men hade trots detta en klart menlig inverkan på plantutvecklingen.

Table I. Mycelium dry weight and final pH on various nitrogen sources after three weeks' incubation in darkness at 25°C. For every treatment comprising four cultures, the average has been given and also the standard error for mycelium dry weight, and significance, where relevant, for comparisons with the controls. The fungus isolates have been classified by ecological type. M = ectotrophic mycorrhiza-formers, L = litter-decomposers and — = fungi of unknown ecological type.

Species	Type	Mycelium dry weight in mg and final pH										
		Control without nitrogen	Ammonium tartrate	Potassium nitrate	Potassium nitrite	Acetamide	Diethylamine	D-glucosamine	Glycine	L-asparagine	L-proline	Pyridine
Agaricus campestris	L	10.6±0.8	38.1±0.6 ***	6.9±0.7	no growth	46.4±1.1 ***	14.1±0.4	10.0±0.9	37.2±0.4 ***	29.7±1.1 ***	10.2±1.6	11.0±0.9
C 267		4.4	5.8	4.5		4.0	4.1	4.3	6.0	6.7	5.1	4.8
Amanita citrina	M	3.3±0.7	3.4±0.4	no growth	»	5.0±0.6	6.7±0.6 *	2.9±0.4	3.1±0.7	no growth	1.9±0.5	2.3±1.8
C 274		5.6	5.4			5.3	4.5	5.2	5.4		6.0	4.8
Amanita muscaria	M	4.2±0.5	26.7±0.6 ***	6.8±0.4 *	»	20.9±0.8 ***	8.2±0.5 **	3.6±0.4	9.6±0.7 ***	»	4.9±0.4	3.6±0.4
C 4		5.4	3.9	4.7		4.2	5.4	5.1	4.5		5.4	4.9
Amanita muscaria	M	4.7±0.7	33.5±1.7 ***	no growth	»	17.9±1.0 ***	2.6±0.3	5.8±0.7	13.5±0.8 ***	12.1±1.9 *	5.7±0.8	3.5±0.6
C 256		5.4	3.6			4.9	5.8	5.3	5.4	4.7	6.6	4.8
Amanita rubescens	M	4.7±0.8	12.8±0.6 ***	7.3±0.6 *	»	6.8±1.4	7.6±1.2	2.4±1.2	2.6±0.4	2.0±0.7	2.8±0.5	not tested
C 6		5.6	4.4	6.3		5.2	5.3	—	5.5	5.6	6.2	
Boletus aeruginascens	—	4.2±0.3	21.2±2.3 ***	3.4±0.1	»	3.5±0.4	8.0±0.4 ***	5.1±0.3	6.5±1.0	no growth	4.4±0.6	2.4±0.5
C 264		5.3	4.2	5.0		5.0	4.8	4.3	5.5		6.7	5.0

Table I. ***(continued)***

Species	Type	Mycelium dry weight in mg and final pH										
		Control without nitrogen	Ammonium tartrate	Potassium nitrate	Potassium nitrite	Acetamide	Diethylamine	D-glucosamine	Glycine	L-asparagine	L-proline	Pyridine
Boletus badius	M	4.0±0.2	23.3±2.6 ***	9.0±0.7 ***	no growth	7.7±1.3 *	8.4±0.6 **	8.0±0.8 **	3.1±0.5	no growth	3.0±0.3	not tested
C 8		5.7	4.0	6.1		5.1	3.4	5.1	5.6		6.2	
Boletus badius	M	4.7±0.3	41.2±1.3 ***	7.5±1.1 *	»	13.9±1.1 ***	7.9±0.5	9.1±0.4 ***	3.8±1.0	»	4.6±0.7	2.4±0.3
C 251		5.6	3.4	5.7		5.0	4.9	4.8	5.6		6.4	5.0
Boletus bovinus	M	4.7±1.1	38.2±0.5 ***	20.1±0.3 ***	»	10.2±0.7 **	3.2±0.3	4.1±0.5	8.4±1.2	35.9±0.8 ***	6.6±0.8	not tested
C 15		5.2	3.6	5.5		4.4	5.5	4.7	5.0	4.4	5.6	
Boletus bovinus	M	5.7±0.7	38.5±0.6 ***	17.5±1.5 ***	»	8.9±0.4 **	5.1±0.3	6.1±0.9	6.4±0.2	43.1±2.0 ***	3.8±1.1	5.9±1.2
C 16		5.4	3.3	5.7		4.6	4.9	4.3	5.1	4.2	5.5	4.7
Boletus bovinus	M	4.8±0.6	36.8±0.8 ***	19.6±1.4 ***	»	13.3±1.6 **	4.4±1.1	4.2±0.4	7.1±0.7 *	15.8±1.3 ***	5.1±1.0	3.6±0.8
C 252		5.1	3.6	5.5		4.1	4.5	4.6	4.7	5.5	5.5	4.8
Boletus edulis	M	4.2±0.8	10.0±0.5 **	3.2±0.3	»	no growth	9.0±0.7	4.0±0.7	34.2±6.5 ***	49.7±7.1 ***	1.9±0.5	no growth
C 18		5.6	4.9	5.5			5.2	5.3	6.2	6.4	6.3	
Boletus edulis	M	4.4±0.6	13.0±1.3 **	4.0±0.4	»	»	no growth	no growth	19.9±3.2 **	58.6±0.3 ***	3.7±0.4	»
C 93		5.6	4.8	5.8					6.0	6.6	6.1	
Boletus elegans	M	8.4±0.3	38.9±4.0 ***	37.7±2.3 ***	»	49.5±3.0 ***	12.7±0.7 **	23.6±1.5 ***	6.8±0.8	50.0±4.3 ***	9.2±0.5	6.6±0.5
C 117		5.6	3.8	7.1		5.6	5.3	4.0	5.4	6.0	5.1	4.9

Table I. ***(continued)***

Species	Type	Mycelium dry weight in mg and final pH										
		Control without nitrogen	Ammonium tartrate	Potassium nitrate	Potassium nitrite	Acetamide	Diethylamine	D-glucosamine	Glycine	L-asparagine	L-proline	Pyridine
Boletus granulatus C 19	M	5.4 ± 1.2 5.3	31.3 ± 1.3 *** 3.7	32.8 ± 0.6 *** 6.1	no growth	15.4 ± 1.1 *** 4.2	4.2 ± 0.1 3.4	9.8 ± 0.3 ** 4.4	27.0 ± 1.3 *** 5.1	19.7 ± 0.5 *** 5.1	3.0 ± 0.8 5.4	2.2 ± 0.5 4.7
Boletus granulatus C 240	M	6.0 ± 1.0 3.8	38.1 ± 2.6 *** 3.3	6.7 ± 0.7 4.5	»	10.9 ± 0.4 ** 3.6	2.2 ± 0.4 3.9	4.0 ± 0.5 3.9	12.8 ± 1.2 ** 4.5	9.7 ± 2.4 4.0	5.2 ± 1.4 4.2	3.1 ± 0.8 4.2
Boletus luteus C 81	M	3.5 ± 0.2 5.6	25.2 ± 0.4 *** 4.1	16.5 ± 1.3 *** 5.6	»	12.8 ± 1.0 *** 4.3	1.4 ± 0.4 6.1	7.0 ± 0.5 *** 5.0	30.7 ± 3.9 *** 6.2	36.6 ± 0.5 *** 6.1	6.7 ± 1.0 * 5.6	2.8 ± 1.0 4.9
Boletus luteus C 243	M	3.5 ± 0.5 5.7	29.4 ± 0.5 *** 3.7	23.7 ± 2.6 *** 5.9	»	8.9 ± 1.0 *** 4.9	1.4 ± 0.3 6.0	6.1 ± 0.5 * 5.0	46.0 ± 1.5 *** 5.3	22.9 ± 2.1 *** 5.2	4.9 ± 0.1 * 5.1	3.8 ± 0.7 4.8
Boletus piperatus C 277	—	3.6 ± 0.6 5.6	5.4 ± 0.3 * 5.2	1.5 ± 0.4 5.6	»	6.1 ± 0.3 ** 5.5	5.3 ± 1.5 5.1	4.7 ± 1.0 5.3	1.8 ± 0.7 5.6	10.5 ± 0.7 *** 5.5	4.3 ± 0.8 6.4	2.5 ± 0.5 5.0
Boletus subtomentosus C 30	L	5.5 ± 0.7 4.9	25.3 ± 0.4 *** 6.3	28.9 ± 1.4 *** 5.1	»	33.5 ± 2.2 *** 5.3	9.5 ± 0.7 *** 4.6	8.7 ± 0.8 * 3.9	28.4 ± 1.1 *** 6.8	35.3 ± 1.1 *** 6.1	3.7 ± 0.6 5.0	4.7 ± 0.3 4.8
Boletus subtomentosus C 118	M	4.4 ± 0.3 5.6	5.7 ± 0.4 * 5.1	4.1 ± 0.4 5.7	»	5.7 ± 0.9 5.1	7.7 ± 0.5 ** 5.7	5.5 ± 0.4 5.3	6.6 ± 1.1 5.7	31.9 ± 1.7 *** 5.6	3.2 ± 1.2 5.9	no growth
Boletus variegatus C 35	M	6.1 ± 1.2 4.0	45.0 ± 0.4 *** 3.2	15.7 ± 1.3 ** 4.6	»	11.1 ± 0.9 3.9	4.9 ± 0.3 4.9	7.5 ± 0.3 4.1	39.7 ± 1.7 *** 4.4	15.9 ± 1.8 ** 5.0	4.8 ± 0.4 4.6	not tested

Table I. ***(continued)***

Species	Type	Mycelium dry weight in mg and final pH										
		Control without nitrogen	Ammonium tartrate	Potassium nitrate	Potassium nitrite	Acetamide	Diethylamine	D-glucosamine	Glycine	L-asparagine	L-proline	Pyridine
Boletus variegatus C 79	M	6.1 ±1.2 5.5	43.2 ±0.5 *** 3.1	7.4 ±1.9 5.2	no growth	5.1 ±0.7 4.6	4.1 ±0.3 5.4	8.5 ±0.6 4.6	38.2 ±3.6 *** 4.9	17.8 ±1.6 ** 5.1	1.7 ±1.0 5.4	not tested
Cenococcum graniforme D 10	M	6.6 ±1.5 5.7	29.6 ±0.8 *** 4.3	15.4 ±1.8 * 6.2	»	14.1 ±0.9 ** 5.2	4.5 ±0.9 5.0	7.9 ±0.5 5.4	13.6 ±0.8 ** 5.5	24.9 ±3.3 ** 5.1	4.7 ±0.8 5.6	4.8 ±0.8 4.8
Clitocybe nebularis C 137	L	5.1 ±0.6 5.3	11.7 ±0.6 *** 4.8	2.5 ±1.3 5.7	»	5.7 ±0.6 5.0	6.1 ±0.7 5.1	7.1 ±0.8 4.8	11.4 ±1.1 ** 5.0	15.7 ±0.9 *** 6.0	3.0 ±0.6 5.6	5.2 ±0.9 5.0
Clitopilus prunulus C 45	—	5.5 ±0.7 5.7	12.4 ±1.2 ** 4.5	1.4 ±0.3 5.6	»	4.9 ±0.4 5.3	7.0 ±0.6 4.8	5.8 ±0.9 5.4	7.9 ±1.1 5.9	17.3 ±1.1 *** 6.2	3.5 ±1.0 6.5	4.7 ±0.9 4.9
Hebeloma crustuliniforme C 87	M	4.1 ±0.8 5.2	32.7 ±0.5 *** 4.2	2.2 ±0.7 5.5	»	3.3 ±1.0 5.2	3.6 ±0.6 5.1	4.7 ±0.8 5.4	6.5 ±0.8 5.6	17.6 ±1.0 *** 6.0	2.2 ±0.6 4.4	4.8 ±0.5 5.1
Hygrophoropsis aurantiaca C 245	L	5.6 ±0.8 3.7	41.9 ±0.5 *** 3.5	4.7 ±0.7 3.8	»	25.9 ±0.5 *** 3.3	6.1 ±1.1 4.1	9.7 ±1.7 2.9	46.3 ±1.4 *** 5.2	59.5 ±1.2 *** 4.6	8.7 ±0.3 * 3.7	7.3 ±1.5 4.8
Lactarius deliciosus C 154	M	4.9 ±1.6 5.6	8.2 ±0.9 5.1	4.6 ±0.5 5.4	»	15.1 ±2.0 ** 5.1	5.7 ±0.6 5.6	4.5 ±1.0 5.4	9.8 ±0.8 * 5.6	4.3 ±0.9 5.7	1.2 ±0.1 6.1	no growth
Paxillus involutus C 122	—	5.0 ±1.0 5.3	44.2 ±1.6 *** 3.4	8.6 ±0.8 * 5.5	»	5.9 ±0.5 4.6	3.5 ±0.8 5.0	3.8 ±0.7 4.2	9.4 ±0.5 ** 4.9	10.0 ±0.9 ** 4.6	3.3 ±0.7 5.7	6.7 ±0.7 4.9

Table I. ***(continued)***

Species	Type	Mycelium dry weight in mg and final pH										
		Control without nitrogen	Ammonium tartrate	Potassium nitrate	Potassium nitrite	Acetamide	Diethylamine	D-glucosamine	Glycine	L-asparagine	L-proline	Pyridine
Paxillus involutus	—	3.7 ± 1.0	36.8 ± 1.4 ***	9.3 ± 0.3 **	no growth	6.6 ± 1.3	1.6 ± 0.5	4.6 ± 0.6	21.9 ± 0.9 ***	12.3 ± 1.5 **	2.0 ± 0.4	not tested
C 248		5.6	3.8	6.6		5.3	5.3	5.4	5.0	4.9	5.0	
Pholiota mutabilis	L	3.5 ± 0.8	52.7 ± 0.7 ***	0.5 ± 0.1	»	14.8 ± 1.0 ***	7.4 ± 1.2 *	6.8 ± 0.7	10.5 ± 1.0 **	58.3 ± 1.1 ***	4.5 ± 0.8	3.3 ± 0.8
C 262		4.8	5.2	4.8		4.4	4.2	3.9	4.7	4.7	4.6	4.9
Rhizopogon luteolus	M	6.5 ± 1.4	42.2 ± 0.5 ***	32.5 ± 0.7 ***	»	9.0 ± 1.4	7.8 ± 0.4	5.3 ± 0.2	38.3 ± 2.1 ***	43.9 ± 2.3 ***	1.5 ± 0.2	5.7 ± 0.5
C 282		4.8	3.3	6.2		4.4	4.4	4.3	5.5	5.3	5.0	4.9
Rhizopogon roseolus	M	6.6 ± 0.8	35.6 ± 0.5 ***	4.2 ± 0.2	»	7.9 ± 0.4	6.9 ± 0.8	8.5 ± 0.4	28.9 ± 2.8 ***	5.6 ± 0.7	5.3 ± 0.2	4.7 ± 0.9
C 236		4.8	3.3	4.4		4.1	4.3	4.3	4.2	5.5	4.9	4.9
Stropharia aeruginosa	L	3.2 ± 0.5	21.9 ± 1.3 ***	1.6 ± 0.5	»	28.2 ± 0.5 ***	5.0 ± 0.6	6.1 ± 1.3	5.2 ± 0.2 *	45.0 ± 2.1 ***	3.3 ± 0.0	not tested
C 265		5.1	5.4	4.9		4.1	4.5	4.5	4.9	4.1	5.5	
Tricholoma imbricatum	M	5.3 ± 0.3	no growth	1.9 ± 0.4	»	7.2 ± 0.3 **	7.0 ± 1.2	6.3 ± 0.3	3.8 ± 0.9	8.3 ± 0.7 *	3.3 ± 0.5	no growth
C 223		5.5		5.5		4.7	4.7	4.5	4.5	4.6	6.8	
Tricholoma nudum	L	5.2 ± 0.7	16.7 ± 0.8 ***	4.5 ± 1.2	»	4.2 ± 0.4	5.0 ± 0.4	5.5 ± 0.4	13.3 ± 0.7 ***	22.1 ± 2.0 ***	3.7 ± 1.1	2.4 ± 0.8
C 225		5.5	4.0	5.4		4.6	4.4	5.2	4.6	5.1	4.8	5.0
Tricholoma pessundatum	M	7.8 ± 0.6	39.6 ± 0.4 ***	36.4 ± 1.1 ***	»	38.4 ± 0.4 ***	13.1 ± 1.6 *	42.5 ± 1.2 ***	32.0 ± 1.7 ***	47.1 ± 0.7 ***	8.5 ± 0.5	no growth
C 226		5.4	5.1	7.0		7.7	4.5	2.5	7.1	6.6	4.8	

Table I. ***(continued)***

Species	Type	Mycelium dry weight in mg and final pH										
		Control without nitrogen	Ammonium tartrate	Potassium nitrate	Potassium nitrite	Acetamide	Diethylamine	D-glucosamine	Glycine	L-asparagine	L-proline	Pyridine
Mean value for:												
Mycorrhiza-formers	M	5.1	28.9	14.5	—	13.3	6.0	8.1	17.4	26.1	4.2	3.7
Litter-decomposers	L	5.5	29.8	7.1	—	22.7	7.6	7.7	21.8	37.9	5.7	5.7
Fungi of unknown ecological type	—	4.4	24.0	4.8	—	5.4	4.9	4.8	9.5	16.9	3.5	4.1
Total		5.1	28.4	11.7	—	14.0	6.2	7.6	17.2	26.9	4.3	4.4