Variation in *Frankia* strains isolated from *Alnus* root nodules.

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Abbreviations

cm	centimetre
cm^3	cubic centimetre
^oC	degrees centigrade
d.w.	dry weight
f.w.	fresh weight
g	gram(s)
ha	hectare
kg	kilogram
l	litre
LSD	least significant difference
µg	microgram
µmol	micromole
ml	millilitre
mm	millimetre
min.	minute
nm	nanometre(s)
nmol	nanomole
p.p.m.	parts per million
p.s.i.	pounds per square inch
r.p.m.	revolutions per minute
S.E.	standard error
s.p.	species provenance
W	watts

Statistical levels of significance

*	$p < 0.050$
**	$p < 0.010$
***	$p < 0.001$
NS	not significant

Summary

Several techniques and media were compared for their effectiveness in the isolation of *Frankia* from nodules of *Alnus*. The most successful technique was based on differential filtration and was used further to isolate *Frankia* from nodules of *Alnus* growing on different sites in Britain. All isolated strains gave rise to sp^- nodules, even when isolated from sites known to contain sp^+ nodules. Strains did, however, vary morphologically and in their carbon nutrition. Differences were also shown in the ability of strains to effect symbiotic nitrogen fixation in nitrogen-free culture. Comparisons with crushed nodules and strains from The Netherlands and North America showed that British strains were as effective and in some cases more effective than foreign strains. In homologous associations nodule specific activity remained relatively constant and differences in strain effectivity were mainly due to differences in plant nodule productivity. In heterologous associations differences were due more to changes in nodule specific activity and nodule growth remained relatively constant. Plants that fixed most nitrogen during the growth period were not those with nodules of highest specific activity. The most effective associations were homologous associations which combined good nodule growth with satisfactory specific activity. Strain effectivity and its determinants were shown to be different in *A. rubra* from different provenances. Nodules from all plants evolved little hydrogen and all demonstrated uptake of hydrogen, probably due to the presence of an efficient uptake hydrogenase, indicating a high relative efficiency. Differences in nodule specific activity were not, therefore, due to differences in relative efficiency.

Field observations showed that infection or spread of *Frankia* seems to be inhibited in some peats. This suggests that soil type may influence the symbiosis. This was confirmed in a glasshouse experiment to examine the effect of soil type, host plant genotype and *Frankia* inoculum source on the symbiosis. The largest effects were observed between different soils; in peat growth was particularly poor and many plants failed to nodulate at all. Differences in growth were also observed with different *Frankia* sources and host plant genotype and interactions were evident between all 3 factors. Further experiments with aqueous extracts from soils and *Frankia in vitro* showed that although extracts from mineral soils were always stimulatory, peat extracts could be either inhibitory or stimulatory. Evidence was obtained which suggests that some strains may be adapted to peat sites.

The prospects for field inoculation of *Alnus rubra* with selected strains was examined. Glasshouse experiments to determine competition between indigenous sp^+ strains and introduced sp^- strains suggest that the introduced *Frankia* competed successfully for infection of new *A. rubra* roots with strains already present in the soil. In a further experiment the benefits of inoculation with selected *Frankia* in the nursery were clearly shown. Although differences in nodule specific activity were similar to those shown under combined nitrogen-free conditions the relative order of effectivity was changed due to soil effects on infectivity and nodule growth. It is clear that, as with the *Rhizobium* symbiosis, further efforts to isolate and identify *Frankia* strains which are capable of high rates of symbiotic nitrogen fixation and which show adaptation to particular environmental conditions should result in better prospects for improved tree growth.

1.0.0 Introduction

1.1.0 Occurrence

Nitrogen fixation is an essential component of the nitrogen cycle, and is the result of the incorporation of gaseous nitrogen into a biologically useful form. All organisms that have so far been confirmed as capable of nitrogen fixation have been prokaryotes (Postgate, 1981). They are either free living or exist in symbiosis with other organisms. Of considerable ecological and economic importance are the symbiotic associations between plants and nitrogen fixing organisms that result in root nodules. Principally, there are two types of association; that between Rhizobium and Leguminosae and that between *Frankia* and non-leguminous (actinorhizal) plants. Both associations have been estimated to contribute considerable quantities of nitrogen to terrestrial ecosystems.

The presence of root nodules on *A. glutinosa* was first reported in 1829, but it was not until 1895 that the role of alder nodules in nitrogen fixation was first demonstrated by Dinger. In a series of experiments on a sandy soil in The Netherlands he showed convincingly that plants with a greater number of nodules generally both grew better and had a higher leaf nitrogen content than others on the same soil, and furthermore, that the removal of nodules both slowed leaf expansion and reduced nitrogen content. However, it was over sixty years before these field observations were supported by experimental work, performed by Hiltner and Nobbe and Hiltner in 1896 and 1904 respectively, who showed that nodulated alders could be grown in a

nitrogen-free nutrient solution. Krebber in 1932 and Roberg in 1934 repeated these experiments and their results confirmed the growth of nodulated plants on nitrogen-free medium (cited Bond, 1967). In his review of 1963 Bond discusses further experiments using similar plant growth techniques to confirm nitrogen fixation by root nodules of *Alnus*, *Myrica*, *Eleagnus*, *Hippophae*, *Shepherdia*, *Casuarina*, *Coriaria*, and *Ceanothus* species. The presence of nitrogen-fixing root nodules ha subsequently been identified in a number of other non-leguminous species.

Microscopic studies by Peklo (1910), Schaede (1933), Pommer (1956), Kappel and Wartenberg (1958), Becking *et al.* (1964) and Gardner (1965) showed the endophyte to be actinomycetal in nature. The genus *Frankia* had been originally defined by Brunchorst in 1886 but was redefined by Becking (1970) for symbiotic actinomycetes which lived in root nodules.

The nitrogen-fixing actinomycete *Frankia* is now known to nodulate a large group of plants all of which, with the exception of two species of *Datisca* are woody (Dawson, 1983). These so called actinorhizal plants are distributed in 24 plant genera (Dixon and Wheeler, 1986) although the number of species known to be actinorhizal is increasing continually and it is expected that many more will be discovered in the future (Akkermans, 1982). Of those identified to date most are located in temperate regions or at high altitudes in the tropics (Dawson, 1983). *Casuarina* is, however, a major exception; originally from Australia it has been taken by man to many other areas and can now be found throughout the tropics and subtropics (Torrey, 1983). The ability to form root nodules capable of fixing nitrogen is usually

a feature of all the species within a genus. In some families, however, nodulation is restricted to a single genus whilst in other families many genera may be nodulated (Akkermans, 1982). Some plant genera such as *Alnus* are almost always nodulated whereas in others nodulation may be highly variable (Wheeler, 1984). Bond (1976) for example, quotes studies carried out in Indonesia where out of 83 trees of *Casuarina equisetifolia* only 3 were nodulated. However,in another location, all 72 trees of *C. sumatrana* examined were found to be nodulated. Lawrie (1982) in a field survey of *Casuarina* in Australia reported that nodules were never found on trees growing more than 70 kilometers from the coast and even trees growing on the coast were not always nodulated. The question arises, therefore, as to whether *Frankia* is always present within soils or whether it is the environmental conditions which do not permit nodulation.

There are conflicting reports in the literature concerning the failure of some soils to nodulate test seedlings; it could be due to the lack of viable *Frankia* or to the existence of soil conditions not conducive to nodulation. Several studies have been described which examine the presence of viable *Frankia* within soils. Rodriquez-Barrueco (1968) for example, in a survey of Scottish soils found that soil from up to 40% of sites did not support nodulation. Similarly, Lawrie (1982) demonstrated that soil samples taken from areas where *Casuarina* was growing but was not nodulated in the field would not support nodulation of seedlings in the laboratory. Nodulation tests performed by Houwers and Akkermans (1981) in a survey of sites in The Netherlands showed that many soils on which alder had not been grown before had either low numbers of *Frankia* or none at all and that this was not due to either soil pH or to other soil factors as inoculation,

in most cases, improved nodulation. Furthermore, Griffiths and McCormick (1984) have reported that *A. glutinosa* seedlings planted in mine soils in Pennsylvania often failed to form root nodules. Such demonstrations of the absence of *Frankia* from soils in which actinorhizal plants are not found support the hypothesis that the population of *Frankia* in soils is mainly determined by nodule decay (eg. Akkermans and Dijk, 1981; Dijk, 1979). However, in contrast Rodriguez-Barrueco (1968) and Huss-Danell and Frej (1986) demonstrated that nodulation of seedlings did occur in soil samples taken from sites where alders, or any other actinorhizal plants, were absent. Clearly, therefore disemination of *Frankia* can occur by other means.

1.2.0 Measurement of nitrogen fixation

Early studies to determine nitrogen fixation in plant material relied on plant growth analyses and subsequent analyses of nitrogen using chemical techniques such as Kjeldahl. Since such techniques are both time consuming and destructive a number of different techniques have been developed to both identify and quantify nitrogen fixation.

One of the most accurate measurements utilises an isotope of nitrogen and in the past both ^{15}N and ^{13}N have been used. The short half-life of ^{13}N, about 10 minutes, has precluded its use in many experiments. In contrast, ^{15}N is stable and has been widely used since 1941 in

measurements of nitrogen fixation (Silvester, 1983) with the first demonstration of nitrogen-fixation in non-legumes by Ziegler and Huser with *Comptonia peregrina* in 1963. There are two main ways in which $^{15}N_2$ is used in studies of nitrogen fixation. Firstly, where plants are exposed to $^{15}N_2$ and the amount of ^{15}N incorporated into the plant is measured and secondly, where the plant is enriched with ^{15}N via the soil and the dilution of plant nitrogen by fixed N_2 gives an indirect measure of nitrogen fixation. The former method is the most reliable and accurate technique for assessment of fixed N_2 over short periods of time (Silvester, 1983). Kohl and Shearer (1980) and Kohl *et al.* (1981) more recently refined this technique after noting differences in the natural abundance of ^{14}N and ^{15}N between recently fixed nitrogen and older soil nitrogen since a small discrimination was known to occur between some biological and chemical reactions (eg. Wada *et al.* 1975). In 1985 Binkley *et al.* investigated the usefulness of this isotope ratio technique for determining the fate of alder-fixed nitrogen. They demonstrated considerable variation in isotope ratios from different sites and although discrimination clearly occurred substantial variability existed. This, together with a number of interacting processes which they identified and discussed, led them to conclude that it would be difficult to use such a technique to demonstrate mechanisms of nitrogen translocation. Its usefulness must, therefore, be limited.

The most widely used technique to demonstrate and estimate nitrogen fixation since its development by Dilworth in 1966, is undoubtedly the acetylene reduction technique. This indirect method relies on the capacity of nitrogenase to reduce not only nitrogen to ammonia but also proportionally acetylene (C_2H_2) to ethylene (C_2H_4). Both

these gases can be analysed rapidly on a gas chromatograph equipped with a flame ionisation detector (eg. Hardy *et al.* 1973; Bergersen, 1970; Postgate, 1981) although in most studies it is C_2H_4 production that is actually measured. The equations representing these reactions are as follows:

$$N_2 + 6e^- + 6H^+ \Rightarrow 2NH_3$$

$$3C_2H_2 + 6e^- \Rightarrow 3C_2H_4$$

The relationship between C_2H_2 and nitrogen reduced would appear, therefore, to be 3:1. However, both in legumes and non-legumes values obtained experimentally often vary considerably from this figure (Hardy *et al.*, 1973; Sprent and Bradford, 1977; Goh *et al.*, 1978). There is now considerable evidence for the involvement of a number of factors, in particular H_2 evolution and uptake, which may affect both the acetylene reduction assay itself and its relationship to nitrogen fixation.

The choice of biological material has been demonstrated to influence nitrogenase activity determined by the acetylene reduction assay. Silver and Mague (1970) showed the age of the nodules to be important in a series of experiments where young nodules of *Myrica cerifera* were found to be more active in acetylene reduction than older nodules. As well as age the portion of the nodule lobe is critical with only the apical 2 mm of alder lobes capable of acetylene reduction Akkermans (1971).In addition detachment of alder lobes considerably reduced acetylene reduction (Akkermans,1971).Similar results were obtained by Wheeler *et al.* (1978) who showed reductions of over 90% in acetylene reduction following detachment of *A.*

glutinosa nodules from the root system. Interestingly, reductions in acetylene reduction by nodules also occurred after removal of either the stems or leaves or after the nodules had been handled. However, Roelofson and Akkermans (1979) reported reductions of only 25-30% in acetylene reduction by *A. glutinosa* nodules following detachment from root pieces. Clearly, therefore, the choice of material is of prime importance when carrying out such assays. In addition criticisms have been made of the systems within which the assays are performed. In these assays the nodule sample is normally incubated within a closed container in the presence of C_2H_2 and air. However, Minchin *et al.* (1983) demonstrated that nitrogenase activity of legume nodules declined in the presence of C_2H_2 and that this decline was due to the cessation of ammonia production. Using a sophisticated continuous flow system for their experiments they argued that closed systems could not be used accurately and would tend to underestimate actual rates of acetylene reduction and thus nitrogenase activity. They found that after a short period of equilibrium acetylene reduction activity of samples peaked rapidly and was then followed by a steep decline. They did, however, concede that if the rate of decline was constant between treatments then closed systems could still be used for comparative purposes. However, they pointed out that observations made by others indicated that variations in magnitude of decline did exist and they suggested that this may explain variations in ratios of C_2H_2:N_2 reduction ratios which have been reported in the literature. Clearly, therefore, results obtained using such closed systems should be treated cautiously and it is normally recommended that C_2H_2 reduction be calibrated against either $^{15}N_2$ or plant biomass measurements (Silvester, 1983). Such observations, together with the influence of environmental variables which are discussed later, go some way to

explaining the variation in the observed to theoretical ratio. However, a much more important reason is that during N_2 reduction approximately 25-30% of the electron flow is diverted into H_2 production and this diversion does not occur in C_2H_2 reduction. Additionally, some organisms are capable of utilising the H_2 through hydrogenase enzymes. These enzymes and their significance are discussed below.

1.3.0 Hydrogenases and their significance

There are two types of hydrogenase enzymes known at the present time; the reversible hydrogenase found in anaerobes and the unidirectional uptake hydrogenase found in nitrogen-fixing organisms (Yates and Eady, 1980).

The presence of uptake hydrogenase in many nitrogen-fixing organisms has been known for some time and although identified in legume nodules by Phelps and Wilson (1941) it was not until 1979 that the enzyme was demonstrated in the nodules of *A. glutinosa* by Benson *et al.*Although Wilson *et al.* (1938) had reported that hydrogen was a specific inhibitor of nitrogen fixation in clover the first evidence that production of H_2 by nodules was linked to nitrogenase activity was provided by Hoch *et al.* (1957) and Hoch *et al.* (1960) and has since been confirmed by others (eg. Dart and Day, 1971). The existence of two separate systems was established by Dixon (1967). Using deuterium and root nodules of *Pisum sativum* he was able to show that one system evolved H_2 via nitrogenase and that another system took up H_2 using a hydrogenase which was O_2 dependant. In addition,

Dixon (1968) showed that this oxidation of H_2 was coupled to the synthesis of ATP. The possible significance of such H_2 uptake systems in nodules of nitrogen-fixing plants was discussed in detail by Dixon (1972) who proposed three possible functions. Firstly, that of oxygen protection - the oxidation of H_2 tending to maintain low levels of oxygen in the tissue. Secondly, the removal of H_2 from the site of N_2 fixation may, he suggested, reduce the established inhibition effects of H_2. Thirdly, the recycling of ATP through hydrogenase may lead to energy conservation. Despite this evidence, however, it was still considered that nitrogenase-dependant H_2 evolution *in vivo* was negligible (Burns and Hardy, 1975) and.presumably that such systems were widespread and of little significance in the efficiency of nodule function. Schubert and Evans (1976) however, demonstrated unequivocally the importance of H_2 uptake systems. They were the first to obtain clear estimates of the amount of ATP wasted when they examined H_2 evolution of root nodules of legumes and non-legumes *in vivo* and convincingly demonstrated the involvement of nitrogenase. They showed that rates of C_2H_2 reduction and H_2 evolution under argon in soya bean nodules lacking a hydrogenase system were equivalent and that the electron flux through the system may be estimated by examining either H_2 evolution under argon and O_2, when no N_2 fixation would occur, or C_2H_2 reduction. Thus the now well known concept of relative efficiency (R.E.) was developed which is calculated as shown:

$$R.E. = 1 - \frac{\text{rate of } H_2 \text{ evolution in air}}{C_2H_2 \text{ reduction or } H_2 \text{ production in } Ar/O_2}$$

This provides a measure of total electron flux that is utilised by the nitrogenase system. Previous work by Bulen *et al.* (1965) had shown that 30% of electron flow through an *in vitro* preparation of nitrogenase from *Azotobacter vinelandii* was utilised in H_2 evolution in air. Values of R.E. which exceeded 0.75, therefore, could be assumed to indicate that some or all of the evolved H_2 was removed by hydrogenase whereas in those cases where R.E. was lower than 0.75 energy was assumed to be wasted as more than the minimum amount of H_2 was evolved. In the survey, out of 19 legumes examined only 2 were found to possess hydrogenase and both contained the same strain of *Rhizobium* - values of R.E. ranged from 0.52 to 0.72. In the actinorhizal plants, however, hydrogenases were more prevalent and nodules of *A. rubra, Purshia tridentata, Eleagnus angustifolia* and *Myrica californica* were all found to evolve only very small amounts of H_2. Only nodules of *Ceanothus velutinus* were found to have a low R.E. In all cases nodules which failed to evolve H_2 were found to take it up when incubated in its presence (Schubert and Evans, 1976) This observed lack of H_2 evolution in actinorhizal plants was in agreement with previous surveys by Moore (1964) and Dart and Day (1971) who also demonstrated little or no H_2 evolution in air as have a number of subsequent studies (eg. Schubert and Evans, 1977). Roelofson and Akkermans (1979) demonstrated high R.E. and H_2 uptake activity of *A. glutinosa* nodules during the summer; in the autumn, however, nodules showed net H_2 evolution and a R.E. of 0.62 to 0.89. The only other report of actinorhizal nodules with a low R.E. was by Sellstedt *et al.* (1986) who demonstrated a R.E. of 0.57 in spore positive nodules of *A. incana*. This high activity of hydrogenase in many actinorhizal nodules contrasts with the low activity of hydrogenase observed in legumes with many rhizobial isolates (Tjepkema *et al.* 1986).

1.4.0 Factors affecting N_2 fixation

1.4.1 Environmental factors

A number of environmental factors have been shown to influence N_2 fixation in both leguminous and actinorhizal plants. Light quality, intensity and duration (eg. Wheeler and Bowes, 1974; Huss-Danell and Sellstedt, 1985) temperature (eg. Dixon and Wheeler, 1983) moisture (Dalton and Zobel, 1977; McNeil and Carpenter, 1979) have all been shown to have an effect on both nodulation and nodule function.

1.4.2 Soil pH and Mineral Nutrition

Numerous studies have been carried out and clear evidence obtained for the effects of pH on both nodulation and nodule function. The limits for nodulation and nitrogen fixation by actinorhizal plants may vary widely and range from pH 3.3 for *Myrica gale* to pH 9 for *Coriara myrtifolia* (Dixon and Wheeler, 1983). *Alnus* usually nodulates best at slightly acid pH and Wheeler *et al.* (1981) defined the best range for nodulation of *A. rubra* to be within pH 4.5 to 6.5. Dixon and Wheeler (1983) ascribe many of the adverse effects of pH to effects on the availability of mineral ions for host plant nutrition. In addition to pH, however, soil texture has also been demonstrated to be important in determining the availability of plant nutrients. Schachtschabel (1963) for example, in a survey of different soil types found the optimum pH for nutrient availability to range from 2.8 to 4.0 for peats to 7.0 to 7.5 for silty loams and clay.

The requirements of actinorhizal plants for mineral nutrients are similar to those for other plant species and have been reviewed by several authors (eg.Gauch, 1972; Epstein, 1972). Of these nutrients Dixon and Wheeler (1983) consider levels of calcium and magnesium to be particularly reduced under *Alnus*. They suggest that this is due to either increased leaching of soil minerals by acid by-products of leaf-litter decomposition or high rates of proton extrusion from the roots due to nodule activity. They also suggest that nitrogen-fixing nodulated species use more phosphorus than plants utilising mineral sources of nitrogen and that this may be particularly obvious in acid soils.

In addition to these basic requirements there are further mineral requirements, necessary for successful nodulation and subsequent efficient nodule function.

Molybdenum is accepted as being essential for plant growth, however, a larger requirement of nodulated plants for this mineral, an essential component of nitrogenase, was originally demonstrated by Becking (1961) and Bond and Hewitt (1961) and is now well established. Although most soils contain sufficient molybdenum, deficiencies often occur and are usually due to adsorption of the element by soil minerals and colloids - this adsorption being closely dependant on pH and greater under more acid conditions (Barrow, 1970). On peat soils, however, deficiencies which appear are most likely due to the retention of molybdenum by insoluble humic acid from the peat, which probably reduces MoO_4^{2-} to Mo^{5+} so becoming fixed and unavailable (Szalay and Szilagyi, 1968).

Cobalt is a specific requirement for vitamin B_{12} which has been identified in actinorhizal nodules and found to be essential for their functioning (Bond and Hewitt, 1962; Kliewer and Evans, 1962; Bond *et al.*, 1965). Although it has not been proven to be essential for higher plants without nodules there are a number of reports in the literature of enhanced growth resulting from its addition to growth medium (eg. Hallsworth *et al.*, 1965). Levels of cobalt are particularly low in highly leached sandy soils derived from igneous rocks or in highly calcareous or peaty soils and availability is further reduced in neutral to alkaline conditions (Mitchell, 1972).

In many cases of poor nutrient availability in higher plants the symptoms can be alleviated by liming; materials such as calcium carbonate, calcium oxide or calcium hydroxide serve both to increase soil pH and the supply of calcium ions (Ca^{2+}), the latter improving soil structure and the former availability of some nutrients (Mengel and Kirby, 1978). Such practices have been applied to actinorhizal plants for example Youngberg and Wollum (1976) and Griffiths and McCormick (1984) both reported increased nodulation of *Ceanothus velutinus* and *A. glutinosa* respectively due to liming.

1.4.3 Combined nitrogen

The effects of combined nitrogen on nodulation and nodule function are now well established and Quispel (1958) both reviews the early reports and provides further evidence. In general, low levels tend to stimulate nodulation resulting in higher nodule numbers, whereas higher levels tend to both reduce nodulation and to impair nodule

function. Stewart (1963) for example, showed an increase in the nodulation of *Myrica* and *Casuarina* species by the addition of low levels of ammonia to the growth medium but a decrease at higher levels. Rodriquez-Barrueco *et al.* (1970) demonstrated significant reductions in the nodulation of *Ceanothus velutinus* and *Casuarina cunninghamiana* by the addition of ammonium and also showed that the proportion of nitrogen provided to the plant by fixation was reduced. Similar results have also been obtained for *Coriaria arborea* by Bond and Mackintosh (1975). Wheeler and McLaughlin (1979) point out, however, that the levels required to inhibit N_2 fixation are high relative to levels of nitrogen usually available under natural conditions.

1.4.4 Other Factors

Numerous observations have been made and experiments carried out which either suggest or show that factors other than those previously discussed may also have some effects on both nodulation and nodule function in actinorhizal plants.

McVean (1956) for example, suggested that alder roots may be particularly sensitive to high concentrations of sulphide which he identified in peats and he observes that the growth of alders was best in waters free from peat acids (McVean, 1962). These studies were largely observational but other evidence is more experimental.

Allelopathic effects were examined by Jobidon and Thibault (1982). They investigated the allelopathic effects of *Populus balsamifera* on

nodulated and unnodulated *A. crispa* seedlings and identified effects on growth, nodulation and N_2 fixation as measured by the C_2H_2 reduction assay. Moiroud and Faure-Raynaud (1983) examined the effects of 4 herbicides on the infectivity of *Frankia* strains for *Alnus*. Of the 4 studied one inhibited nodulation completely. Similar work by Moiroud *et al.* in 1985, using the same herbicides and one fungicide, examined their effects on the growth of *Alnus* and on nodule formation. They demonstrated that at low concentrations the herbicides had no effect on nodulation and although nodulation was inhibited at high concentrations this was due to inhibition of the growth of the host plant. The one fungicide tried, however, had no effect on the *Alnus* seedlings but inhibited nodulation completely. Evidence has also been presented that the activities of other soil microorganisms may also play an important part in the infection process under natural conditions. The first report was by Knowlton *et al.* (1980) who demonstrated that the soil bacterium *Pseudomonas cepacia* could stimulate infection of *A. rubra* by *Frankia* through, they proposed, causing enhanced root hair deformation thus allowing intimate contact between *Frankia* filaments and the root hair wall. In further experiments Knowlton and Dawson (1982) described the interaction of these so called 'helper bacteria' with pH. They found that the effects of inoculation with *P. cepacia* were particularly evident at pH 5 and 5.5 whereas there was little effect at pH 7 and pH 9. Other organisms, however, quite apart from assisting the infection process may actually prevent it through competition with *Frankia* for infection sites. This possibility was convincingly demonstrated by Dijk (1980). He observed root nodules on *A. glutinosa* which had been caused by the soil fungus *Penicillium nigrans* and which were similar in appearance to young *Frankia* induced nodules. Furthermore, mixed inoculum

experiments suggested that they competed for the same infection sites and this led Dijk to propose that the widespread distribution of *P. nigrans* in the soil may be an important determinant of nodulation in the field.

The widespread existence of factors which may influence either nodulation or nodule function and the undoubtedly large number of factors which still remain to be identified provide adequate reasons for caution in both the control of conditions under which experiments are carried out and the interpretation of such results - particularly when drawing parallels to field situations.

1.5.0 Importance of actinorhizal plants in forestry with particular reference to *Alnus*

The significance of actinorhizal plants as pioneers in natural ecosystems is now well established and this subject has been reviewed by a number of authors (eg. Crocker and Major, 1955; Tarrant and Trappe, 1971; Silvester, 1977). Undoubtedly their success can be attributed largely to their ability, in association with *Frankia*, to fix atmospheric nitrogen and thus survive at sites of low nutrient availability, particularly of nitrogen, where other species can not. This competitive advantage may be important even in later stages of succession where fixation rates are normally lower (Becking, 1977). It is no doubt an awareness of these nitrogen-fixing properties that in the past has led to the inclusion of actinorhizal plants in traditional and rotational agriculture. In the highlands of Guatemala, for example, native farmers realise that scattered alder trees allowed

to remain in maize fields increase crop yields (Dawson, 1979) and *Casuarina* was included in traditional rotational agriculture practices in the highlands of New Guinea (Silvester, 1977).This species is currently attracting considerable attention and shows great potential as a forest crop in the tropics and subtropics and as a firewood crop in the Third World (Bostid, 1984)

In more temperate regions, however, *Alnus* has been researched extensively over the past 50 years and has been described by Dawson (1983) as perhaps the most important actinorhizal tree genus. Plantings of several *Alnus* species have been made, in the past, for soil amelioration purposes and *A. glutinosa* has been used extensively in Eastern and Central United States for the revegetation of disturbed lands (Griffiths and McCormick, 1984). The first experimental plantings of this species in the United States were made in 1958 (Dale, 1963; Funk, 1973). Although in 1964 it was estimated that 1.8 million seedlings were being sown in state nurseries and in 1968 the figure had increased to 3.7 million (Funk, 1973) a decade later this figure had declined to 1 million (Fessenden, 1979). *Alnus* species have also been planted widely in Britain, The Netherlands and Germany (Dawson, 1983) and in Britain *A. glutinosa* and *A. incana* accounted for approximately 60% of all plantings on colliery spoil between 1976 and 1977 (Jobling and Stevens, 1980).

Alnus species have also been highly regarded as a forest species in either pure or mixed stands or as part of a rotation system. *A. glutinosa* and *A. rubra* have both been extensively investigated for growth in pure stands and *A. rubra* has been estimated to be probably the highest yielding forest tree (Gordon, 1975). *A. rubra* is also

particularly suitable for growth in pure stands or as part of a rotation due to its rapid juvenile growth and good growth form. Timber harvested from *A. rubra* has excellent working qualities and will both take and maintain a high finish and although in western North America nearly all the wood is used for furniture purposes its uses are diverse and it can also be used for pulp and particle board manufacture (Evans, 1984). In the United States natural stands have been estimated to yield between 15 and 25 tons of above ground biomass per hectare per year without management (Gordon and Dawson, 1979) Together with reports of nitrogen accretion which range from 40 (Newton *et al.*,1968) to 323 (Tarrant and Miller, 1963) kilograms per hectare per year these figures illustrate why in some regions of the United States *A. rubra* is regarded as a major hardwood (Radwan *et al.*, 1984). Apart from traditional forestry plantings in pure stands a number of other uses have been proposed for *Alnus* spp. and include short-rotation forestry (Granhall, 1982; De Bell, 1979) and as a mixture in conventional forest plantations (eg. Tarrant and Trappe, 1971). In pure stands and energy plantations silvicultural systems can be developed fairly readily. In mixed plantings, however, the advantages of N_2 fixation and increased soil nitrogen associated with the 'nurse' species often have to be weighed against possible 'overtopping' by the nitrogen-fixer resulting in less than optimal growth of the main crop species. A number of management systems involving optimal spacings and species have thus been suggested by a number of authors (eg. Red'ko, 1958). Clearly, however, what applies to one site may not apply equally to another. It may, for instance, be more difficult to demonstrate improvements in yield of the main crop species where the site is not nitrogen deficient. Such factors may go some way to explaining the variable assessments which have been made

by various authors of the value of *Alnus* (and other actinorhizal species) in mixed stands. Some studies, for example, using *A. rubra* in mixed stands with *Populus* have demonstrated improvements in tree growth over the first 3 or 4 years by inclusion of the nitrogen-fixing species (De Bell, 1979) whilst others have shown no improvement in growth (Tessier *et al.*, 1984).

Because the natural distribution of *A. rubra* coincides very largely with that of sitka spruce it has been included in a number of trials in Britain on similar sites (Evans, 1984). The sites on which they have been planted are usually upland sites which are often nutritionally poor and relatively exposed. They have been planted in pure stands and all provenances examined so far are generally susceptible to autumn frosts (Lines and Brown, 1982). Origins from high elevations in British Columbia, the Cascade, Coastal and Olympic mountain ranges of North Western America may, however, confer greater frost hardiness and together with hybridisation studies with *A. sinuata* may improve frost resistance (Evans, 1984).

1.6.0 Isolation of *Frankia*

For many years research into *Frankia* was hampered by the inability of researchers to obtain pure cultures of the endophyte. Attempts to isolate the organism had been made from early in this century with the first recorded isolation attempt by Peklo (1910). A great many attempts were made subsequently and these have been reviewed

extensively by Bond (1963) and Baker and Torrey (1979). A large number of the attempts were criticised, however, and many of the supposed successful isolations could not be confirmed (eg. Uemura, 1961; Wollum *et al.*, 1966). Such controversy led to the suggestion by Bond (1967) that isolation attempts should be submitted to critical independent confirmation. One particular attempt by Pommer (1959) from *A. glutinosa* nodules is, however, worth special mention as the characteristics of the isolate he reported are very similar to the currently accepted definition of a *Frankia* species (Lechevalier and Lechevalier, 1979) and that the isolate could produce root nodulation was recently confirmed by Becking (1981). At the time, however, the report attracted much criticism from Quispel (1960) on the basis that the isolation medium Pommer used was too simple. It was almost 80 years after Peklo's first attempt when Callaham *et al.* (1978) using a fairly complicated enzymic digestion technique obtained the first confirmed (Lalonde, 1978) isolation of the endophyte. Subsequently a number of simpler techniques were developed and to date a large number of *Frankia* strains have been isolated from a wide range of actinorhizal species using a variety of different methods which are described below. In principle they all involve 1) the release of endophyte from the nodule by mechanical disruption 2) the removal of contaminants 3) the incubation of the endophyte in selective media.

1.6.1 Serial dilution

This technique is one of the simplest to be used in the isolation of *Frankia* and has been used widely in microbiology for the isolation of eubacteria (eg. Jensen, 1968); it is described in detail by Baker and Torrey (1979). In its simplest form it involves crushing nodules in sterile distilled water and the preparation of a series of

dilutions of the homogenate. Fractions of this homogenate are then inoculated onto pour plates and at optimum dilutions the organism will grow out in the form of isolated colonies, clearly distinguishable from contaminants. This technique and variations using selective incubation media were most widely used in attempts to isolate *Frankia* prior to 1978 (eg. Lalonde *et al.*, 1975; Quispel and Tak, 1978) and more recently in the isolation of *Frankia* from root nodules of *Casuarina equisetifolia* by Diem *et al.* (1982).

1.6.2 Sucrose density centrifugation.

This technique was first used by Baker *et al.* (1979) and relies on the differential separation of the various components of nodule tissue throughout the gradient due to their different densities. Basically, a crushed nodule suspension is prepared, filtered crudely and applied to a discontinuous sucrose density gradient of 60%, 45% and 30% (w/v) sucrose. The gradient is then centrifuged at 100,000g for 3 hours and different fractions collected from the tube which are used to inoculate pour plates. Burggraaf *et al.* (1981) using this technique reported the successful isolation of *Frankia* from *A. glutinosa* and *M. gale* with a simpler single 60% (w/v) sucrose solution, centrifuged at 25,000g for only 30 minutes. Another modification of the technique by Baker and O'Keefe(1984) permitted the isolation of *Frankia* from soils. However, these isolates proved to be ineffective on re-inoculation of host plants.

1.6.3 Microdissection.

Although used in many earlier unsuccessful or unconfirmed attempts at isolation (eg. Pommer, 1959; Costa and Rodriquez-Barrueco, 1976) it was not until 1979 that it was used successfully by Berry and Torrey

(1979) to isolate *Frankia*. The technique involves the surface sterilisation of nodules, after which they are sliced into transverse sections and the endophytic hyphae or vesicle clusters are removed with the aid of dissecting needles. Associated cell debris is then removed by both centrifugation and filtration and the endophyte material used to inoculate pour plates. There are no reports of its recent use in the literature.

1.6.4 Sterilisation with osmium tetra-oxide (OsO_4).

One of the major difficulties encountered in the isolation of *Frankia* is the presence of contaminating organisms - particularly where they may have similar nutritional requirements and can not be inhibited by the use of selective media. This technique utilises the powerful sterilant OsO_4 to remove such organisms. It was first used to successfully isolate *Frankia* by Lalonde *et al.* (1981) and is now widely used (eg. Hafeez *et al.* 1984; Normand and Lalonde, 1982). The technique involves the sterilisation of the outermost layers of the nodule by immersion in a 3% solution of OsO_4 for a short period, after which the nodules are fragmented and either incubated in liquid medium or, in modifications of the technique by Burggraaf (1984), plated onto agar medium. One disadvantage of the technique is the toxicity of OsO_4 and consequent caution which must be exercised in its use (Wheeler, 1984).

1.6.5 Differential filtration

This relatively simple technique allows vesicle clusters to be plated at high density with only few contaminants (Tjepkema *et al. 1986*). *It was first used by Benson (1982) to isolate Frankia* from nodules of *Alnus* and has subsequently been applied with considerable success

(Malcolm *et al.* 1985; Wheeler *et al.*, 1986). Put simply it involves the sequential filtering of nodule homogenates through nylon mesh of different pore size and the thorough rinsing of the homogenate; fractions of the retained suspension are used to inoculate pour plates.

Despite the relatively rigid categorisation of the techniques above, in practice many are altered substantially from the original description of the technique and some investigators may even use combinations of a number of different techniques.

1.6.6 Isolation medium.

In conjunction with these techniques a number of different isolation media have been employed. The medium may be either liquid or solidified by the addition of agar. Liquid media are easier to use but solid media have the advantage of spatially separating possible contaminants, thus reducing the chances of the potential *Frankia* isolates being overrun before they are of sufficient size or stage of development to be removed. Those *Frankia* strains which have been isolated so far appear to be relatively undemanding in their nutritional requirements (Wheeler, 1984). Media which have been commonly used are QMOD (Lalonde and Calvert, 1979), *Frankia* broth (Baker and Torrey, 1979) and a medium based on sodium propionate (Shipton and Burggraaf, 1982). There are reports by Quispel *et al.* (1983) however, of a requirement of some strains from The Netherlands for root-extract lipids during isolation, although many strains did not retain this requirement once they were in liquid culture. Quispel *et al.* (1983) and Burggraaf (1984) discuss in detail the possible nature of these extracts.

1.7.0 Variation among *Frankia*

The existence of a large number of cultures of *Frankia* strains from various actinorhizal host and their characterisation has produced, over recent years, a substantial amount of information on the variability which exists between different strains. In some cases attempts have been made to identify characteristics which are relatively constant between strains, or groups of strains, so as to assist in their classification and provide clues as to their common ancestry. In others attempts have been made to identify variation between strains in order to assess the genetic diversity which exists within the genus - sometimes with the aim of manipulation. The main areas of variation which have been identified are summarised and their recognised implications discussed below.

1.7.1 Morphology

Most *Frankia* strains isolated to date are characterised in culture by the formation of branched septate hypha and sporangia containing non-motile spores (e.g. Akkermans, 1982; Callaham *et al.* 1978; Lechevalier and Lechevalier, 1979; Baker *et al.* 1980); thus conforming to observations previously made on actinorhizal endophytes *in vivo* (Dijk and Merkus, 1976; Gardner, 1965). Under nitrogen limited cultural conditions they all form specialised structures from terminal swellings of hyphae, which are termed vesicles, and these cultures will reduce N_2 (Tjepkema *et al.* 1980; Meesters *et al.* 1985) The vesicles have for some time been proposed as sites of nitrogenase activity (e.g. Tjepkema *et al.* 1980; Gauthier *et al.*, 1981) and this was recently confirmed by Meesters *et al.* (1985) using immuno-

electrophoresis-techniques. The structure and physiology of the vesicles has been examined and have been demonstrated to be made up of complete multilaminate walls (Torrey and Callaham, 1982) and to provide a mechanism of oxygen protection for nitrogenase (Murry *et al.* 1984).

Despite these general features, however, considerable variation has been observed to occur both between individual strains under similar conditions and within the same strain under different cultural conditions. Hyphal size varies within individual strains and may be dependant on the maintenance medium (Berry and Torrey, 1979). Some strains may produce pigments whilst others do not. A strain from *M. pensylvanica*, Mpl1 is described as green, for example, and a strain from *Casuarina* P1 as red whilst another strain ArbN4$_b$ from *A. rubra* is described as white (Baker, 1982). Some strains may also produce pigmented spores; for example, spores of AirI2, a strain isolated from *A. rugosa* are black.

Vesicle formation has been shown to be dependant on the concentration of combined nitrogen in the culture medium and only occurs when it is at low levels (Tjepkema *et al.* 1981). An exception to this, however, was recently reported by Meesters *et al.* (1985) in a strain from *Casuarina* where vesicles continued to form in relatively high concentrations of ammonium chloride (NH_4Cl) although no nitrogenase activity was detected. Vesicles *in vitro* are usually rounded (Tjepkema *et al. 1986). In vivo*, however, they may occur in a number of different shapes. Vesicles in *Alnus, Hippophae, Elaeagnus, Shepherdia, Colletia* and *Discaria* are spherical. In *Purshia, Rubus, Ceanothus, Dryas* and *Chamaebatia* pear shaped and in *Comptonia, Myrica,*

Cercocarpus, *Datisca* and *Coriaria* are club shaped to filamentous (Tjepkema *et al.* 1986). Such variation given the *in vitro* shape would appear to be host dependant and the demonstration by Lalonde (1979) that inoculation of *Comptonia* with the strain CPI1 resulted in club shaped vesicles whereas inoculation of *A. glutinosa* with the same strain resulted in spherical vesicles confirms this. The most interesting variation in this respect, however, appears in nodules of *Casuarina*. Although strains from *Casuarina* have been observed to produce vesicles in culture (e.g. Diem *et al.* 1982; Lancelle *et al. 1985*) none have been observed *in vivo* (Tyson and Silver, 1979; Berg, 1983).

Spore formation too is influenced by cultural conditions (Lalonde and Calvert, 1979; Perradin *et al.* 1983; Baker and Torrey, 1980; Burggraaf, 1984) although virtually all strains will form spore containing sporangia under the right conditions. *In vivo*, however, two types of nodules can be distinguished on the basis of spore formation. Those in which spores are abundant and intracellular (sp^+) and those in which they are rare and intercellular (sp^-) (Dijk and Merkus, 1976); this difference is proposed by Normand and Lalonde (1982) VandenBosch and Torrey (1985) and Dijk (1978) to be the result of differences in endophyte genotype. To date both types of nodule have been observed in a number of *Alnus* species, *Myrica gale* and *Comptonia peregrina* and sporangia have been observed in nodules of *Casuarina*, *Hippophae* and *Ceanothus* (Tjepkema *et al.* 1986).

It is also possible that some strains may produce other structures in addition to those normally identified with *Frankia*. A strain isolated from *Casuarina* for instance has been reported to produce torulose

chains of spore-like cells from vegetative hyphae and which grow out readily under favourable conditions; it was proposed that they may complement sporangia as survival structures (Diem and Dommergues, 1985).

1.7.2 Carbon nutrition

Frankiae have been shown to be capable of the utilisation of a number of different carbon sources for growth in culture. Such experiments are, however, difficult to perform due to long induction periods which have been reported by Benson and Hanna (1983) to vary from 2 to 5 weeks. They are usually carried out both as a means of characterising strains in culture and as a means of obtaining information on the way in which *Frankia* may utilise different carbon sources and their pathways of metabolism.

Carbon sources used by Frankia for growth are often quite diverse. The most effective are often fatty acids such as propionate and acetate, derivatives of fatty acids such as Tweens, intermediates of the tricarboxylic acid cycle like succinate or malate and organic acids such as pyruvate (Tjepkema *et al.* 1986). TCA cycle, glyoxylate cycle and gluconeogenic enzymes have been demonstrated in AvCI1 (Blom and Harkink, 1981). Although some strains will grow on sugars such as glucose growth is often relatively poor (eg. Shipton and Burggraaf, 1982) and the inability of many strains to use such sources at all (eg. Shipton and Burggraaf, 1982; Tisa *et al.* 1983) has been interpreted by Lechevalier and Ruan (1984) as sometimes being due to the lack of a carbohydrate transport system. The identification of trehalose and the likelihood that it was being metabolised in pure

cultures of HFPArI3 by Lopez *et al.* (1983) led to speculation that enzymes of glycolysis must be involved, at least in some strains. Confirmation that such pathways do exist came two years later when Lopez and Torrey (1985) reported enzymes of glycolysis in both pure cultures and vesicle cluster preparations. This led Tjepkema *et al.* (1986) to speculate that most strains, if not all, probably possess enzymes for carbohydrate metabolism.

Although there is, therefore, considerable information concerning *in vitro* use of carbon sources very little is known of the carbon sources utilised by *Frankia* and their metabolism in symbiosis and only a small number of studies have been performed. These few studies have been carried out using vesicle cluster suspensions.

The presence of TCA enzymes was reported by Huss-Danell *et al.* (1982) in such preparations from nodules of *A. glutinosa* and *H. rhamnoides* but enzymes involved in irreversible steps in glycolysis were not found. Furthermore, Akkermans *et al.* (1983) demonstrated that vesicle clusters from *Datisca* could respire succinate and that O_2 uptake in the presence of succinate could be enhanced by the addition of ADP. This suggests that succinate or some other organic acid could be utilised by *Frankia*, in symbiosis, at least in some strains (Wheeler, 1984). In addition Akkermans *et al.* (1983)also showed that vesicle clusters from nodules of *A. glutinosa* and *H. rhamnoides* would also uptake O_2 when supplied with glutamate, malate and NAD and suggested that this could indicate that reducing equivalents were transported to the vesicle clusters via the malate-aspartate shuttle.

Such studies with vesicle clusters have also identified the need to exercise caution in extrapolating from results obtained in pure culture. Huss-Danell *et al.* (1982) failed to identify activity of glyoxylate cycle enzymes isocitrate lyase and malate synthetase in vesicle clusters extracted from *A. glutinosa* nodules produced as a result of inoculation with AvCI1. Since these enzymes are known to be present in AvCI1 when grown in culture medium with Tween 80 as a carbon source they suggested that these enzymes are repressed in symbiosis. Similarly, Akkermans *et al.* (1983) identified the presence of damaged plant mitochondria within isolated vesicle clusters and pointed out that because of this it is difficult to draw conclusions from such experiments although, at the same time, they point out that the contribution of damaged plant mitochondria to the respiration of vesicle clusters will probably be small.

1.7.3 Infectivity

Differences in the infectivity of some *Frankia* strains for nodulation of host plants are well established. Prior to the successful isolation of *Frankia* by Callaham *et al.* (1978) infectivity tests were performed using crushed nodules as a source of inoculum. Using these preparations a large number of different experiments were carried out in order to determine compatibility of the endophyte between different host plant genera (eg. Bond, 1963 and 1967; Rodriguez-Barrueco and Bond, G. 1968). Demonstrations of incompatibility barriers led Becking (1970, 1974) to propose that all nitrogen-fixing actinomycetes capable of forming mutualistic symbioses with higher plants should be classified into the genus *Frankia* and suggested that

both morphological features and incompatibility should be used as a criterion for sub-division. It soon became apparent, however, that such criteria were inadequate. Rodriquez-Barrueco and Bond (1976) for example, demonstrated that inoculation of *M. gale* with crushed nodules from *A. glutinosa* resulted in nodulation but *A. glutinosa* did not form nodules when inoculated with crushed nodules from *M. gale*. Miguel *et al.* (1978) however, demonstrated that crushed nodules from *M. gale* would nodulate *A. glutinosa*. They also showed that crushed nodules from both *Alnus* and *Hippophae* could infect *Elaeagnus* seedlings whilst crushed nodules from *Alnus* would not infect *Hippophae*. Such evidence showed the unsuitability of these criteria for classification.

The development of isolation procedures enabled pure cultures to be used in tests of infectivity and this provided further evidence. CpI1 isolated by Callaham *et al.* (1978) from *Comptonia peregrina* also formed effective nodules on *M. gale* and *M. cerifera* and on six *Alnus* species investigated (Akkermans and Dijk, 1981) but not on *Casuarina*, *Elaeagnus* and *Ceanothus* (Baker and Torrey, 1979) - in contrast to the observations by Miguel *et al.*

Infectivity can, therefore, be influenced by the host plant species but clearly there are no clear divisions which can be made. In general it is possible on the evidence to date to define 3 groups: *Alnus*, *Myrica* and *Comptonia*; *Hippophae*, *Coriaria* and *Ceanothus* and finally *Casuarina*. However, exceptions have been reported. Diem *et al.* (1982) for example, isolated a *Frankia* strain from *Casuarina* which failed to nodulate the host but nodulated seedlings of *Hippophae*.

Other strains have been isolated which are non-infective (eg. Baker *et al.* 1980).

Not only may the infection potential of an endophyte source be either positive or negative it may also vary depending upon the host plant even when the endophyte is from the same host genus. There is relatively little information available on the relative infectivity of different *Frankia* strains, although Burggraaf (1983, 1984) showed that the growth stage of the culture was important, that different strains from *A. glutinosa* may infect different sections of root and that whilst some strains may form many nodules on a host plant others may only form a few. Crushed nodule homogenates from sp^+ nodules of *A. glutinosa* were reported by Houwers and Akkermans (1981) to be 1000 times more infective than homogenates from sp^- nodules.

1.7.4 Effectivity

Differences in the ability of some *Frankia* strains for effectivity in nitrogen fixation have been observed. In the most extreme example nodules are formed but no fixation occurs. These ineffective strains have been isolated by a number of investigators (Baker *et al.* 1980) and Lie *et al.* (1984) showed, using ineffective mutants of AvCI1, that this was not due to lack of nitrogenase. Of particular interest, however, to those concerned with optimising the growth of actinorhizal plants are the reported differences in the extent to which some *Frankia* strains will effect growth in symbiosis.

Dawson and Sun (1981) and Dillon and Baker (1982), using acetylene reduction by nodules as a measure of effectivity, both identified significant interaction between the plant host and endophyte source and demonstrated different degrees of compatibility between *Frankia* strains from different species. Normand and Lalonde (1982) examined the effectivity of a large number of *Frankia* strains from two *Alnus* species which they assessed by measurements of plant height. They found that, in general no differences in effectivity existed between strains isolated from an individual provenance and that although some differences did exist between some strains isolated from different provenances the host origin of the strain did not appear to be important. They identified the most important variable in effectivity to be the spore nature of the strain *in vivo*. Strains isolated from sp^+ nodules were, in general, only approximately 70% as effective as those isolated from sp^- nodules. These results support those of previous work by Hall *et al.* (1979) and Maynard (1980) who also reported reductions in the effectivity of sp^+ strains using crushed nodules. In another study Carpenter *et al.* (1984) examined the effectivity of *Frankia* strains isolated from two provenances of *A. rubra* and *A. sinuata* when inoculated onto two clones of each species, using both plant growth and acetylene reduction as a means of assessment. They reported that both host genotype and endophyte may affect the effectivity of the symbioses. Their data is, however, difficult to interpret and sometimes contradictory. In another study Simon *et al.* (1985) inoculated three *A. glutinosa* clones with 4 *Frankia* strains, 3 sp^- and one sp^+. They reported superior performance as a result of inoculation with the sp^- strains and significant differences in growth were observed with different clones. Finally Reddell and Bowen (1985) used crushed nodule inoculum from *Casuarina*

from different locations and species to inoculate two *Casuarina* species and demonstrated differences in the effectivity in symbiosis of nodules formed as a result of inoculation with the nodules from the different sources and an additional effect of species.

The reasons for differences in effectivity are not clear. One possible explanation is differences in R.E. as determined by hydrogen evolution and discussed earlier. An alternative explanation for the differences between sp^+ and sp^- strains was, however, reported by VandenBosh and Torrey (1984). They found that although sp^+ nodules of *C. peregrina* and *M. gale* evolved only small amounts of hydrogen and had therefore high R.E. values, compared to sp^- nodules they had a high CO_2 to ethylene evolution ratio and thus a high respiratory cost for nitrogen fixation.

1.8.0 Outline of Thesis

In Britain *A. glutinosa* is the only native *Alnus* species. Other alders, including the exotic *A. rubra* are now planted on a number of sites. *A. rubra* is grown in Britain from seed and is presumably nodulated by *Frankia* indigenous to British soils. The question arises, therefore, whether nodulation is with fully compatible *Frankia* strains and whether inoculation with *Frankia* strains from the natural distribution range of *A. rubra* in North Western America, could improve nitrogen fixation and growth of this species in Britain. Previous work by Wheeler *et al.* (1981) has shown considerable variation in the specific activity of nitrogen fixation by *Frankia* from British soils. However, they used only soils as inocula. In this study, a method was

identified which permitted routine isolation and characterisation of *Frankia* from both the indigenous *A. glutinosa* and the introduced *A. rubra*. The growth responses of *Alnus* to inoculation with *Frankia* isolated from Britain and North Western America were evaluated. Furthermore, attempts were made to examine the relative importance of the effects of soil type and host genotype on the symbioses. Finally, the effects of the inoculation in the nursery of *A. rubra* with a number of strains were evaluated and compared to crushed nodule inoculum.

2.0.0 Materials and Methods

2.1.0 Isolation of *Frankia*

2.1.1 Nodule collection, storage and characterisation

Frankia were isolated from nodules obtained from Britain, North America, The Netherlands and Sweden. Nodules from British sites (Table 1) were, where possible, collected on the same day as isolation. Where this was not practicable nodules were stored in the dark in a sealed polythene bag, within either a cooled vacuum flask or polystyrene container, and on arrival at the laboratory were transferred to a coldroom maintained at 4°C. Nodules from Juneau, Alaska and from McNab's Farm, Vancouver Island, British Columbia were received by airmail from Drs. B. Borman and M. Cannell respectively. Nodules from Umea, Sweden were received from K. Huss-Danell on plants maintained in Perlite. Nodules from Britain and The Netherlands used in the comparison of isolation methods described in 1.2.7, were stored at -20°C for 2 to 4 days after harvest or obtained from 3 month old greenhouse plants grown in water culture and inoculated with crushed nodule inoculum.

The presence or absence of spore producing cells in nodule samples was determined by light microscopy of transverse or longitudinal sections, stained if necessary with Fabil reagent (Noel, 1964). Sections (10 to 20μm) were cut at 150μm intervals through each of 5 randomly

Table 1: Details of sites from which nodules were collected for isolation of *Frankia* and fruits were harvested for use in experiments.

Site	Location[1]	Soil description	Soil pH
Milngavie	NS543767	Loam	5.0
Balmaha	NS409920	Loam	3.4
Tentsmuir	NO500265	Sandy soil in dune belt	6.7
Rumster 9[1]	ND084467	Deep peat, highly organic	3.5
Rannoch Moor	NN446312	Sandy, edge of loch	4.8
Woodhall	NT278646	Coal waste	ND
McNab's Farm	British Columbia	Loam	4.5
Lennox[2]	NS606762	Loam	5.1
Shin 93[2]	NC495216	Deep peat, highly organic	3.6
Falstone 6[2]	NY657928	Peaty gley	ND
South Yorks. 9[2]	SE014197	Discontinuous iron pan with localised pockets of podzolised brown earth	3.5
Wykeham 116	TA945890	Iron pan on low calcareous grit	3.2
Bush[3]	NT246635	Loam	5.8
Wauchope 9[2]	NY523022	Deep peat, highly organic	3.5
Shin 65[2]	NC531220	Deep peat, highly organic	3.8
Shin 45[2]	NC327043	Deep peat, highly organic	3.6
Tumble	SN545115	Waste site	ND
Southport	SD301131	Sandy soil, coastal dune belt	7.4

[1] Ordnance survey grid reference (British sites).
2 Forestry Commission Plantation.
3 Forestry Commission Nursery.
ND Not determined.

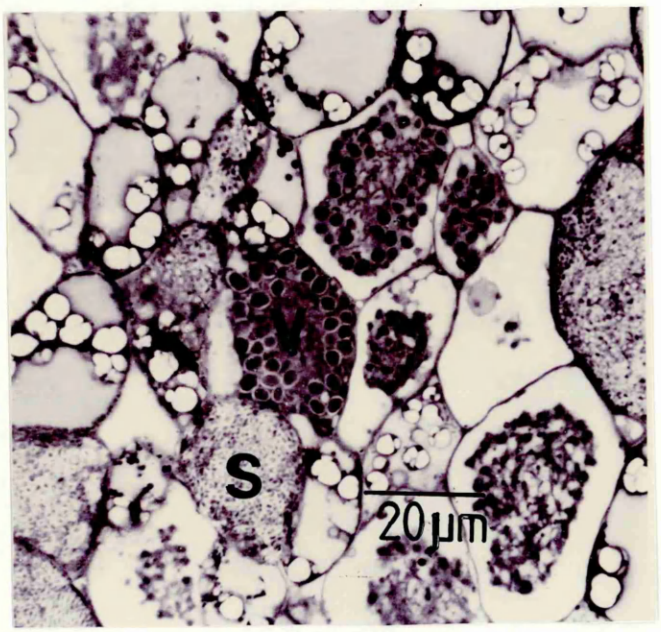
selected nodule lobes using a Leitz (Kryomat) freezing microtome operated with a stage and blade temperature of -10°C. Spore cells were easily recognisable by light microscopy (Plate 1) and stained bright red. For all British sites soil characteristics were evaluated on site and, in the case of Forestry Commission experimental sites, from Forestry Commission records. Samples, from the upper 10cm of the soil horizon, were also collected and transferred back to the laboratory as described for the nodules. Immediately upon return to the laboratory pH measurements were made (1:1 distilled water) using a Howe Model 6030 pH meter.

2.1.2 Isolation techniques

Microdissection

The method employed was essentially that described by Lalonde *et. al.* (1981) but 3% chromium trioxide was used to sterilise the outer layers of the nodules, in place of osmium tetroxide. Where appropriate, manipulations were carried out aseptically within a laminar flow hood. Nodule clusters were first detached from the roots and washed thoroughly in tap water to remove any soil. A number of nodule lobes were then excised, using a sterile scalpel, and transferred to a sterile test tube. The lobes were washed 5 times by agitating in 10ml sterile distilled water and decanting, and were then surface sterilised for periods of either 1, 3, or 5 minutes by agitating with 5ml chromium trioxide. After the appropriate time period the sterilant was removed by decanting and the lobes rinsed thoroughly 5 times by agitating in 10ml sterile distilled water. A single lobe was transferred to a sterile petri dish and the outer cortex removed using

Plate 1: Transverse section of a nodule of *A. glutinosa* showing cells containing vesicles, v and spores, s of *Frankia*. *Photograph courtesy* of C.T. Wheeler.


S
20µm

sterile forceps and a sterile scalpel. A longitudinal section was then cut from each lobe and a single slice transferred to 15ml liquid medium in a sterile test tube. The media used were FMC+P containing casamino acids and sodium pyruvate; Qmod containing glucose, yeast extract, Bacto-peptone and lecithin; Bu containing sodium propionate and P(+) containing glucose and asparagine together with an aqueous nodule extract (see Appendix 1 for full details of media). The tubes were sealed with Nescofilm (Nippon Shoji Kaisha Ltd., Osaka, Japan), incubated in the dark at 27°C and regular observations made. Any growth from a nodule slice was examined by transferring the slice to a sterile petri dish and examining under a light microscope.

Differential filtration

The method employed was based on that described by Benson (1982). Where appropriate procedures were carried out aseptically within a laminar flow hood. Nodule clusters were first detached from the roots and washed thoroughly in tap water to remove any soil. Single lobes were then selected, great care being taken to avoid decayed lobes, and 1 to 2mm cut from the tips of a number, normally 10 to 12, using a sterile scalpel. The tips were then transferred to a sterile test tube and surface sterilised by agitating for 5 minutes with 10ml 20% laundry bleach. The bleach was removed by decanting and the lobe tips thoroughly rinsed 5 times by agitating in 20ml sterile distilled water. The lobe tips together with 5ml liquid isolation medium were then transferred to a sterile Potter Elvehjem tissue homogeniser and the plunger operated until the material was disrupted; usually about 20 times. The media used were as for the previous technique with the

additional media P(-) containing glucose and asparagine but no aqueous nodule extract and FB containing yeast extract, dextrose and casamino acids (see Appendix 1 for full details of media). The resulting homogenate was filtered sequentially through firstly a 53μm and secondly a 20μm nylon mesh screen (Plastok Associates, Birkenhead). The screens were held in place on cut nylon syringe barrels with nylon O-rings and the whole assembly sterilised before use by autoclaving for 35 minutes at 120°C 15 p.s.i. A 10μm screen was also used initially but this was discontinued in later isolations because of the rapidity with which the screen became blocked with residue and the success of the other 2 screens in retaining *Frankia*. The residue collected on the 50μm screen was washed 7 times with 5ml liquid isolation medium and the residue collected on the 20μm screen 4 times with 15ml isolation medium. The residues were removed from the screens with a sterile Pasteur Pipette by resuspending in liquid isolation medium, transferred to a sterile test tube and made up to 5ml with liquid isolation medium. Vesicle counts were usually performed on each suspension using a haemocytometer and the suspensions used to inoculate pour plates (Petri dishes; 9cm, double vent) at various concentrations. Ten to 15 plates were poured with each agar isolation medium, approximately 30ml per plate, and incubated in the dark at 27°C. Regular observations were made using a light microscope. After 8 weeks the number of colonies presumed to be *Frankia* on the basis of their morphological characteristics were recorded for each isolation plate together with details of colony form and pigmentation. In some cases hyphal diameter was also measured.

2.1.3 Culture of isolates

Colonies were normally removed from the isolation plates when quite large; often 1 to 2mm in diameter and 3 to 4 months after isolation. A single colony was carefully dissected from the isolation plate using a sterile scalpel and as much agar as possible removed using a sterile petri dish as a cutting surface. The colony was then homogenised with 5ml BuCT medium (see Appendix 1 for details of composition) in a sterile Potter Elvehjem homogeniser and the remaining suspension transferred to a 100ml flask containing 50ml BuCT medium. Three colonies of each colony type observed in a particular isolation / isolation medium were treated in this way. Following observation of growth of the isolate the culture was routinely sub-cultured by first disrupting the isolate by sucking and expelling through a syringe needle (Yale, 21G) several times and transferring an aliquot of the suspension to fresh BuCT medium. If the characteristics of each of the three colonies of a particular morphological type observed on the isolation plate remained similar following sub-culture, then only one of the culture lines was selected for continued maintenance. Culture lines showing differences were all maintained. In all cases each culture was allocated an acronym according to the nomenclature described in Appendix 2 and all sub-cultures and medium changes recorded. Where the colony failed to grow in BuCT or its rate was relatively slow single colonies were transferred to the medium on which they had previously been isolated.

2.1.4 Infectivity of isolates

Presumptive *Frankia* isolates were examined for their ability to infect both *A. rubra* and *A. glutinosa*. *A. rubra* fruits were harvested from Lennox Forest and *A. glutinosa* fruits from Milngavie (for details of sites see Table 1) and stored in a refrigerator prior to use.

Seeds were germinated in seed trays in Perlite (ratio 3:1, standard grade:superfine grade) moistened with a nutrient solution consisting of full strength Crones-N and Hoaglands A-Z micronutrients (for composition of nutrients see Appendix 3). Ten litres of Perlite were moistened with 2 litres of nutrient solution and thoroughly mixed; the pH was adjusted to 6.0 (1:1 distilled water) with 2N sulphuric acid. Seedlings were transplanted 35 to 40 days after sowing, that is after the formation of the first true leaf and before the emergence of the second, into plastic bags of capacity 1.25 litres (Transatlantic Plastics Ltd.) filled with Perlite moistened as before. Twelve to 15 seedlings of each species were transferred; approximately 4 to each bag.

Isolates were freed of medium by centrifuging at 2500 r.p.m. and 4°C for 10 minutes (Mistral M.S.E. 2L) resuspending with distilled water and repeating. The mycelia were then disrupted by sucking up and expelling through a syringe needle (Yale, 21G) and the resulting suspension used to inoculate the seedlings. A series of non-inoculated controls were also set up. The seedlings were given maintenance doses of combined nitrogen in the form of Fison's Liquinure (for composition see Appendix 3) during the first 4 weeks after

inoculation. The seedlings were maintained in a heated greenhouse supplemented with 400W mercury vapour lamps (above 15°C, 16 hour photoperiod) and watered regularly with tap water. After 16 weeks seedlings were examined for nodulation and evidence of fixation (ie greening, strong growth).

2.1.5 Stability of colony morphology

The colony morphology of strains on agar isolation plates was compared to the morphology of colonies when grown in a single agar medium, BuCT.

Frankia strains, all of which were routinely maintained previously on BUCT, were thoroughly rinsed with a pH 6.8 buffer (1 g l^{-1} K_2HPO_4 and 0.67 g l^{-1} NaH_2PO_4) using a sterile Analytical Filter Funnel (Nalgene Labware, type AF) *in vacuo* to remove all traces of the medium. The culture was then resuspended in buffer and dispersed by passing repeatedly through a syringe needle (Yale, 21G). Aliquots (2ml) were added to 5 petri dishes (9cm, double vent) for each strain examined, approximately 30ml BuCT agar medium added, which had been cooled previously to 35 to 40°C, and the plate swirled to aid mixing.

Plates were sealed with Nescofilm and incubated in the dark at 27°C. After 8 weeks the colonies which had grown were examined using a light microscope and a description of the colony form recorded.

2.1.6 Comparison of isolation techniques

A number of techniques which have been used successfully for the isolation of *Frankia* were compared in a series of experiments carried out at the University of Leiden, The Netherlands in collaboration with T.A. Quispel and A.J.P. Burggraaf. Three techniques were compared using nodules of *A. glutinosa* collected from the field at Diemen, Hoogmade and at Balmaha (see Burggraaf, 1984 for descriptions of the Diemen and Hoogmade sites and Table 1 for description of the Balmaha site) and from greenhouse plants inoculated with crushed nodules from Diemen and Balmaha (see Burggraaf, 1984) using a number of different media. Nodule sections were cut from lobes of all field nodules with a microtome and examined with a light microscope for the presence of spore cells. The techniques applied were carried out as follows and where appropriate aseptically within a laminar flow hood.

(A) Differential filtration

Nodule clusters were first removed from the roots and any soil removed by washing thoroughly in tap water. The lobe tips (2 to 3mm) were then excised using a razor and sterilised as follows:

(a) soaked for 5 minutes in an evacuated soap solution,

(b) agitated for 5 minutes in 70% ethanol,

(c) rinsed in sterile distilled water,

(d) agitated in a 1.5% solution of osmium tetroxide for 1 to 2 minutes,

(e) rinsed in sterile distilled water.

The lobes were then homogenised with a glass pestle and 5ml of the appropriate isolation medium in a test tube and the remaining homogenate sequentially filtered through the differential filtration apparatus as described in 2.1.2. Equal aliquots of the 20μm filtrate were applied to a number of isolation plates for each medium used.

(B) Sucrose density fractionation

The technique employed was based on that used for *Frankia* isolation by Baker *et al.* (1979). Nodule lobes were first prepared, excised and sterilised as in (A). A number of lobes were then homogenised in sterile distilled water and 1 to 2ml of this homogenate applied to a single sucrose layer of 60% (w/v) and centrifuged for 30 minutes at 2100g. The sucrose layer was sampled with a sterile Pasteur Pipette and the solution applied equally to a number of isolation plates for each medium used.

(C) Nodule fragment

Nodule lobes were first prepared, excised and sterilised as in (A). A number of lobes were then broken into small fragments by grinding with a glass pestle and 5ml sterile distilled water in a test tube. The resulting fragments were rinsed in sterile distilled water until the solution was clear and 6 fragments applied to a number of isolation plates for each medium used with sterile forceps.

The isolation plates were double layered. The first layer comprised 1% agar in 20ml of the appropriate medium which was allowed to solidify. The top layer was 5ml of 0.8% agar, cooled to 44 to 46°C. Five or six plates of each of the following media compositions were poured for each comparison: M-N/Cas containing casamino acids; M-N/Cas/TL containing casamino acids and a total lipid extract; P-N/Cas containing sodium propionate and casamino acids and P-N/Cas/TL containing sodium propionate, casamino acids and a total lipid extract (see Appendix 1 for details of media). Where total lipid (TL) extract was used this was only included in the top layer. Plates were incubated in the dark at 28°C and inspected at regular intervals using a light microscope.

Comparisons of the three methods were made using: (a) field collected root nodules of *A. glutinosa* from Diemen (sp^-) and Balmaha (sp^+) and (b) three month old nodules from greenhouse grown *A. glutinosa* inoculated with sp^+ nodules from Balmaha. An isolation using the differential filtration method was also made from 3 month old nodules from greenhouse grown *A. glutinosa* inoculated with sp^- nodules from Diemen.

Assessments

Identification of *Frankia* was made on the basis of morphological characteristics. Counts of percentage vesicle clusters or nodule pieces with hyphal outgrowths were made together with colony counts.

2.2.0 Characterisation of Strains

2.2.1 Determination of spore occurrence in nodules

Fruits of *A. rubra* and *A. glutinosa* were germinated, and seedlings prepared and inoculated as described in 2.1.4. The expression of spore production by individual strains in each host / strain combination was determined by examining sections cut as described in 2.1.1 from 5 lobes of nodule clusters from 3 randomly selected plants 20 to 24 weeks after inoculation. This time interval is greater than was found necessary for *Frankia* to develop spores in nodules of *Alnus* by C. van Dijk (personal communication) and by VandenBosch and Torrey (1985).

2.2.2 Utilisation of carbon sources *in vitro*

A number of strains were examined for their ability to (a) utilise a range of carbon sources and (b) grow on a medium containing no combined nitrogen source. The medium composition was as described for Bu in Appendix 1 but different carbon sources (5g l^{-1}) were substituted for sodium propionate and ammonium chloride was omitted in one case. Tween 80 was added later to the appropriate tubes to give a final concentration of 0.5g l^{-1}. All carbon sources and media were filter sterilised and dispensed using a Pharme-Aide Fluid Dispensing System (American Pharmaseal Laboratories, Glendale, CA 91209) fitted with a Millex GS, 0.2µm filter cartridge. *Frankia* strains, all of which were maintained previously routinely on BUCT, were rinsed thoroughly with a

pH 6.8 buffer (1g l^{-1} K_2HPO_4 and 0.67g l^{-1} NaH_2PO_4) using a sterile Analytical Filter Funnel (type AF, Nalgene Labware) *in vacuo* to remove all traces of the medium. The culture was then resuspended in buffer and disrupted by passing repeatedly through a syringe needle (Yale, 21G). Aliquots (1ml) were added to each of 3 tubes (3cm x 15cm) containing 10ml of medium with the appropriate carbon and nitrogen sources. Tubes were covered with aluminium caps (Oxoid), sealed with Nescofilm and maintained in the dark at 27°C under non-shaking conditions.

After 14 weeks *Frankia* growth within each tube was assessed visibly against the control tubes and scored as follows:

0	no growth,
1	traces,
2	clearly visible growth,
3	mycelia completely cover base of tube,
4	mycelia occupy at least 25% of the tube contents.

In cases where no growth was observed, and with the exception of those tubes inoculated with ArI3, ArI4, ArI5 or Agn1Cl2, where Tween 80 had been added or omitted as an initial treatment, Tween 80 was added to give a concentration of 0.5g l^{-1}.

After a further 14 weeks, growth in the tubes supplemented with Tween 80 was assessed against the remaining control and scored as described previously. To those tubes, excepting controls, which still showed no growth 2ml of BuCT were added. As all the strains had been maintained previously on this medium the continued viability of the strain could be confirmed by observation of subsequent growth.

2.2.3 Effectivity of crushed nodule inocula for nodulation and nitrogen fixation in *A. rubra*

Nodule sources and inoculum preparation

Nodules were obtained from the following sources:
1)Timberlands Farm, Vancouver Island (V.I.), 2)McNabs Farm, V.I., kindly provided by M. Cannell and 3)Corvallis, Oregon, collected by C.T. Wheeler and maintained on *A. rubra* grown in water culture for 5 years prior to use in these experiments. Nodules from Timberlands Farm and McNabs Farm sources were identified as being sp^- and nodules obtained from the Corvallis source identified as being sp^+ after examination of sections cut as described in 2.1.1.

Nodule lobes were separated and surface sterilised by agitating in 95% methanol for 5 minutes. Lobes were rinsed thoroughly in distilled water and the upper 2 to 3mm of the tips removed with a sterile razor. The tip material (1g) was then ground thoroughly in a mortar and pestle and diluted with distilled water to a final volume of 2l. This homogenate was used to inoculate the seedlings.

Fruit germination, seedling inoculation, plant growth and harvest

A. rubra fruits were collected in November 1983 from two trees growing in a pure stand at Lennox Forest. Fruits were sown thinly in four circular containers measuring 45cm diameter and 19cm deep containing Perlite moistened with nutrients as in 2.1.4. Approximately forty days

after sowing, that is after emergence of the first true leaf but before emergence of the second, the seedlings were inoculated by pouring equal quantities of the relevant nodule homogenate over the entire surface of the containers.

The fruits were germinated and plants grown in a heated greenhouse (above 15°C, photoperiod 16 hours) with daylight supplemented initially with 400W mercury vapour lamps. During the first three weeks after inoculation small measured quantities of Liquinure were applied equally to all seedlings. Seedlings were sampled regularly and examined for nodulation, using a dissection microscope where necessary, until swellings typical of nodules were first observed on the roots and this time was recorded. Ninety one days after nodulation 30 seedlings were selected randomly from each inoculated group and transferred to 1.25l plastic bags (see 2.1.4 for supplier) containing Perlite moistened as before. The plants were watered regularly with tap water.

Plants were harvested 225 days after nodulation. Eight plants were randomly selected from each inoculated group. Plant height was measured, root, shoot and nodule dry weights were determined and the number of nodules recorded for each plant. Ratios of root to shoot and plant to nodule dry weight were calculated. Dry weights were of material after being dried in an oven at 80°C to constant weight. Five nodule lobes from 3 of the remaining plants from each inoculated group were examined as in 2.2.1 in order to determine spore production by *Frankia* in the nodules. Determinations of total nitrogen were carried out on dried material from each treatment. The material from all the harvested replicates for each treatment was combined, ground to a fine

powder and mixed, dried for a further 48 hours in an oven at 70°C and assayed for nitrogen content using a semi-micro Kjeldahl procedure as follows. Duplicate samples (0.1 to 0.3g) were digested with 1.5 to 2ml concentrated H_2SO_4 containing 30g l^{-1} salycilic acid and 0.2 to 0.3g catalyst (catalyst: potassium sulphate, 1g; copper sulphate, 1g and mercuric oxide 1g) until clear. Ammonia was then released from the digest by the addition of 40% NaOH in a Markham Still, captured in 2% boric acid with a mixed indicator of methyl red and bromocresol green and titrated with 0.01N HCl.

Statistical analysis

Analysis of Variance was carried out on measured and calculated parameters of growth. Where this analysis showed significant ($p < 0.05$) treatment effects the significance of difference between means was determined using Duncan's Multiple Range Test (Duncan, 1955) at $p < 0.05$. Analysis of Variance was carried out using the statistical package MINITAB (Statistics Department, Pennsylvania State University, Release 81.1) run on either the University of Glasgow Computing Services VME or the University of Edinburgh EMAS computers. Duncan's Multiple Range Test was carried out as described in Ridgeman (1975).

2.2.4 Determination of effectivity of *Frankia* strains for nodulation and nitrogen fixation

(A) Water culture grown plants

Preparation and quantification of inocula

Frankia strains ArI3, ArI4, ArI5, AUCI1, and CPI1 gratefully received from Drs. A. Berry, J.G. Torrey, and D. Baker were used as inocula in this experiment.

All strains were maintained routinely on Bu medium and subcultured 56 days prior to use. Frankia were harvested by filtration on a 0.3μm membrane filter (Millipore, 47mm diameter) *in vacuo* using a Millipore apparatus and were rinsed thoroughly with distilled water. The residual *Frankia* was resuspended in distilled water using a Pasteur Pipette, transferred to a graduated 10ml centrifuge tube and centrifuged at 2500 r.p.m. and 4°C for 10 minutes (M.S.E., Mistral 2L) after which the packed cell volume was recorded. The supernatants were decanted and the pellets each made up to 5ml with distilled water. The resulting suspensions were homogenised thoroughly using a Potter Elvehjem homogeniser and diluted equally with distilled water.

Fruit germination, seedling inoculation, plant growth and harvest

A. rubra fruits were harvested in November 1982 from trees growing in a pure stand at Lennox Forest. Fruits were sown thinly in seed trays containing Perlite moistened with a nutrient solution as described in 2.1.4. Forty days after sowing, that is, after emergence of the first true leaf but before the emergence of the second, the seedlings were carefully transferred to 2 litre water culture pots filled with a nutrient solution consisting of distilled water containing Crones-N 1.8g l^{-1}, Hoaglands A-Z micronutrients 1ml l^{-1} and Liquinure 0.105ml l^{-1} (see Appendix 3 for composition of nutrients). Initially 7 seedlings were positioned in each pot; held in place within holes drilled in a plastic pot cover with a ring of rubber tubing and cotton wool. After 20 days the seedlings were thinned, leaving the tallest 3 per pot, and the nutrient solution replaced with a solution of distilled water containing Crones-N 1.8g l^{-1} and Hoaglands A-Z micronutrients 1ml l^{-1}. Each pot was inoculated with a *Frankia* suspension equivalent to 0.033ml packed cell volume of a particular strain. Five pots ie a total of 15 seedlings were inoculated with each strain.

The plants were maintained in controlled environment growth cabinets (16 hours light, 21°C; 8 hours dark, 19°C). Light was provided by a mixture of daylight and warm-white fluorescent tubes which provided 79±3.5µmol m^{-2} s-1 light (n=4), measured using a Quantum Measuring System (Skye Instruments Ltd.) at seedling height. The pots were arranged randomly within the cabinets; co-ordinates selected from random number tables. The water culture pots were topped up regularly with distilled water.

Sixty five days after inoculation plants were harvested. Plants were inspected for nodulation and the percentage of plants nodulated calculated. Plant dry weight was determined after drying in an oven at 80°C to constant weight. The nitrogen content of harvested plants was determined using a semi-micro Kjeldahl procedure as described in 2.2.3. Plants harvested from individual pots were grouped together for analysis.

Statistical analysis

Analysis of Variance was carried out on plant dry weight measurements using the statistical package MINITAB. Where significant ($p < 0.05$) effects of treatments were indicated using this technique the significance of the difference between means was determined using Duncan's Multiple Range Test (see 2.2.3 for details of both techniques).

(B) Perlite grown plants

Sources of inocula and experimental design.

The *Frankia* strains used as inocula in these experiments were ArI3, ArI4, ArI5, and Agn1C12 (kindly provided by Dr. A. J.P. Burggraaf) and a number of the strains isolated from *A. rubra* and *A. glutinosa* at Glasgow and described in this Thesis (see Results). Crushed nodule inoculum was from *A. rubra* growing in a pure stand at Lennox Forest, near Glasgow (see Table 1 for site details).

The number of strains to be assessed was too large to permit comparison between inoculated plants at one time. The growth of plants inoculated with different strains was compared, therefore, in a number of separate batches; batches 1 to 3 containing plants inoculated with ArI4 to provide a basis for comparison between these batches. Strain comparisons were carried out in 6 batches. The strains compared in individual batches together with the inoculated species and provenance are indicated below:

Batch 1: *A. rubra*, Lennox; ArI4, ArI5, ArI3, 1.2.5[P+], 1.2.5[Q](b), 1.1.1[BuC], crushed nodules.

Batch 2: *A. rubra*, Lennox; ArI4, 1.1.2[Q], 1.1.4[F], 1.1.5[F], 1.1.8[Bu], Agn1C12.

Batch 3: *A. rubra*, Lennox; ArI4, 1.2.15[Bu], 1.2.23[Q](b), 1.2.13[Bu], 1.2.13[Q], 1.2.20[Bu], 1.2.19[Q], 1.1.14[Q].

Batch 4: *A. rubra*, Lennox; 1.2.5[Q](b), 1.2.5[Q](b) and crushed nodules, crushed nodules.

Batch 5: *A. rubra*, Lennox; 1.1.4[F], 1.1.5[F], 1.1.8[Bu]. *A. glutinosa*, Milngavie; 1.1.4[F], 1.1.5[F], 1.1.8[Bu].

Batch 6: *A. rubra*, Prince Rupert provenance; ArI4, 1.2.19[Q], 1.2.23[Q](b). *A. rubra*, McNab's Farm provenance; ArI4, 1.2.19[Q], 1.2.23[Q](b) . *A. rubra*, Menzies Bay provenance; ArI4, 1.2.19[Q], 1.2.23[Q](b).

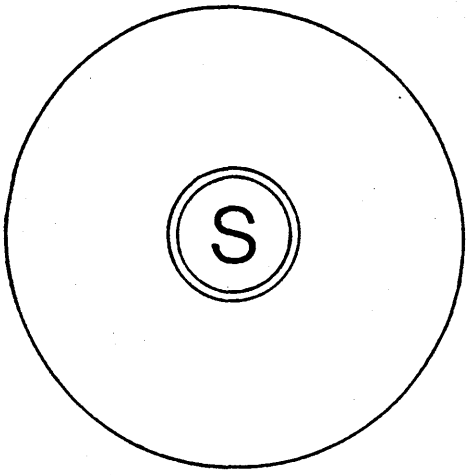
Preparation and quantification of *Frankia* strain inoculum

All strains were maintained routinely on BuCT medium and subcultured 28 days previously. Strains were harvested by centrifuging at 2500 r.p.m. and 4°C for 10 minutes (M.S.E., Mistral 2L) and decanting the medium. The resulting pellet was resuspended with distilled water and centrifuged as described previously and the supernatant decanted. This was repeated 3 times to ensure that no traces of the medium remained. The final pellet was diluted to 10ml with distilled water and the culture dispersed by sucking it up into and expelling it sequentially through a 21 guage syringe needle (Yale) 5 times and a 25 guage needle 3 times using a 10ml disposable syringe. The resulting homogenate was diluted to 20ml with distilled water. Assays of protein content were made on a 4ml sample after sonication at full power (Ultra-sonics 180S) in a water cooled sonication chamber (Figure 1) for 60 seconds. Preliminary experiments had shown that no further protein release occurred after sonication of samples under these conditions for 50 seconds (Figure 2). Protein determinations were made on duplicate 1ml samples after mixing with 2ml of a protein reagent (Pierce Assay Reagent, Product number 23200) based on the dye Coomassie Blue G250 (Bradford, 1976). Sample absorbance was measured at 595nm in a spectrophotometer (M.S.E., Spectroplus D), after standing 10 minutes at room temperature for full colour development, and protein yields were determined after comparison with Bovine Serum Albumen (Sigma Chemical Company Ltd.) standards (see Figure 3 for example of standard curve). Homogenates were diluted to a suitable volume with distilled water to ensure the concentration of *Frankia* protein was 5.25$\mu g\ ml^{-1}$ except where indicated.

Figure 1: Diagram of Sonication Chamber.

Samples were sonicated at full power for 60 seconds, before determination of protein yield. Figures are means of 3 samples.

A



B

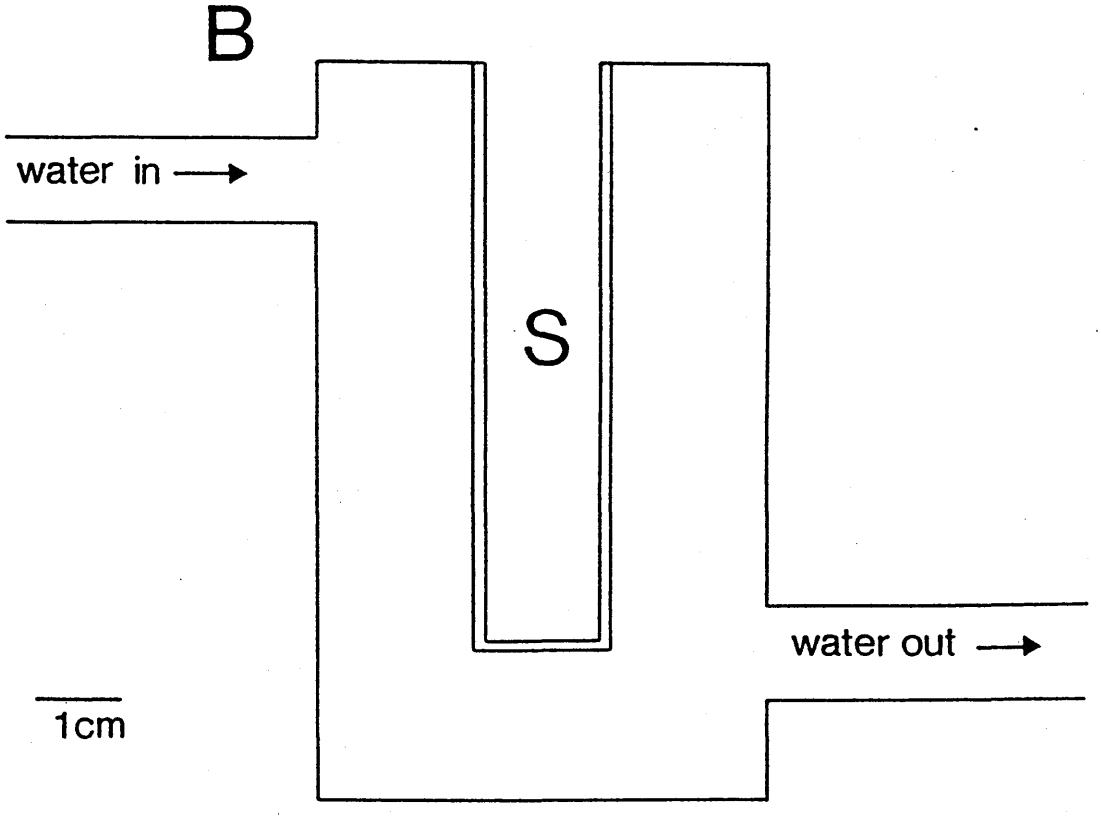
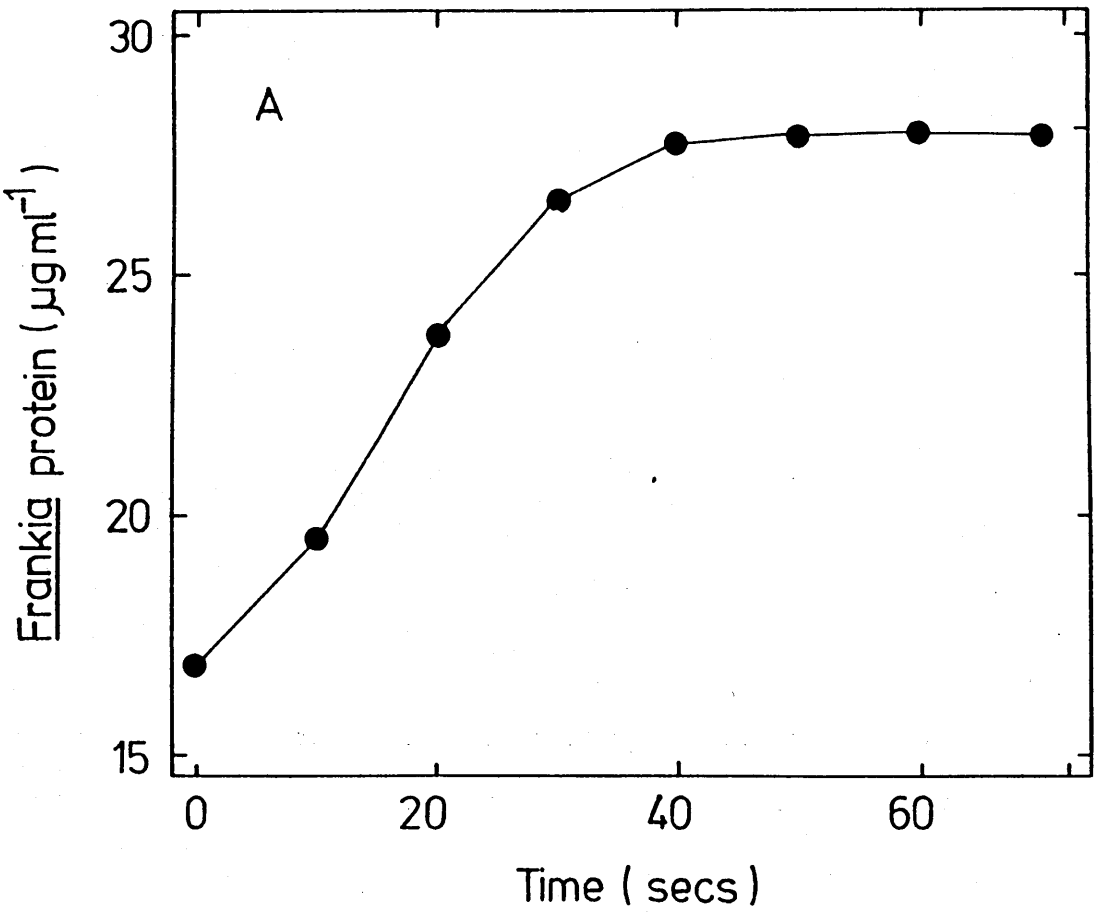


Figure 2: Time course for solublisation of protein released from *Frankia* by sonication in A) a cooled sonication chamber and B) a cold shoulder cell.

Figures are means of 3 samples. S.E. < 2μg.


A
30
25
20
15
0
20
40
60
Frankia protein (µg ml⁻¹)
Time (secs)

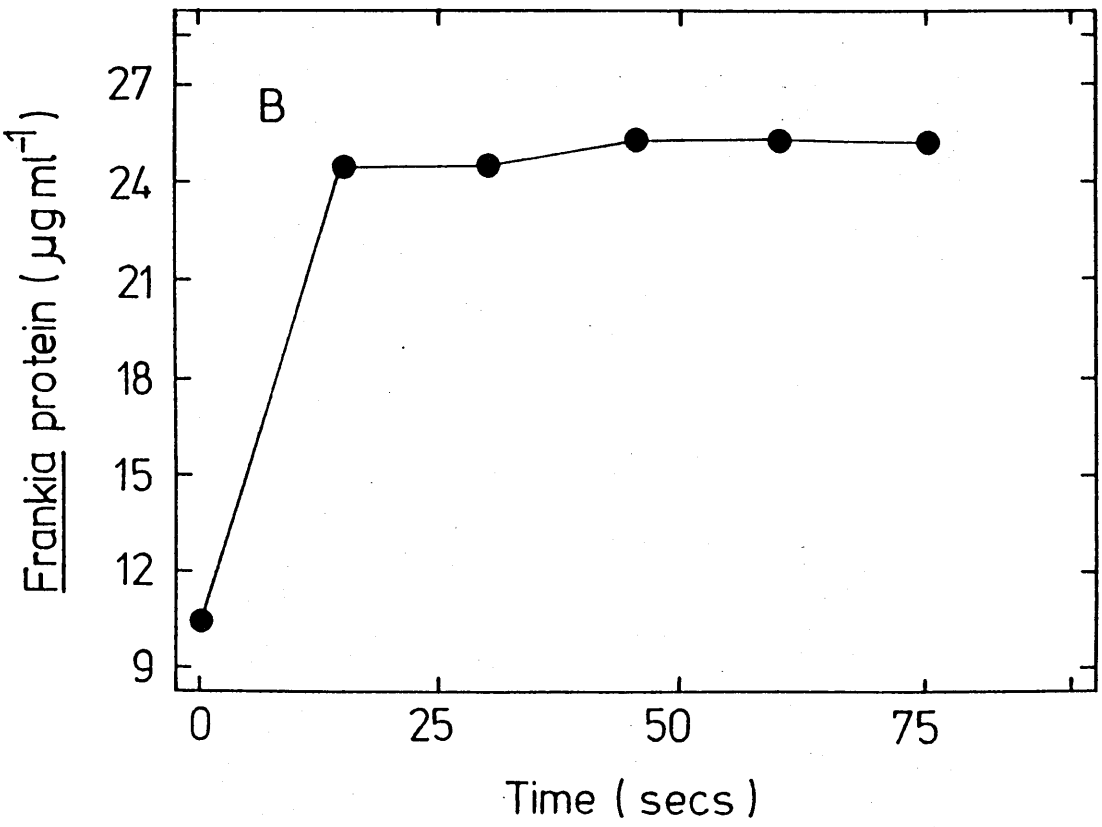
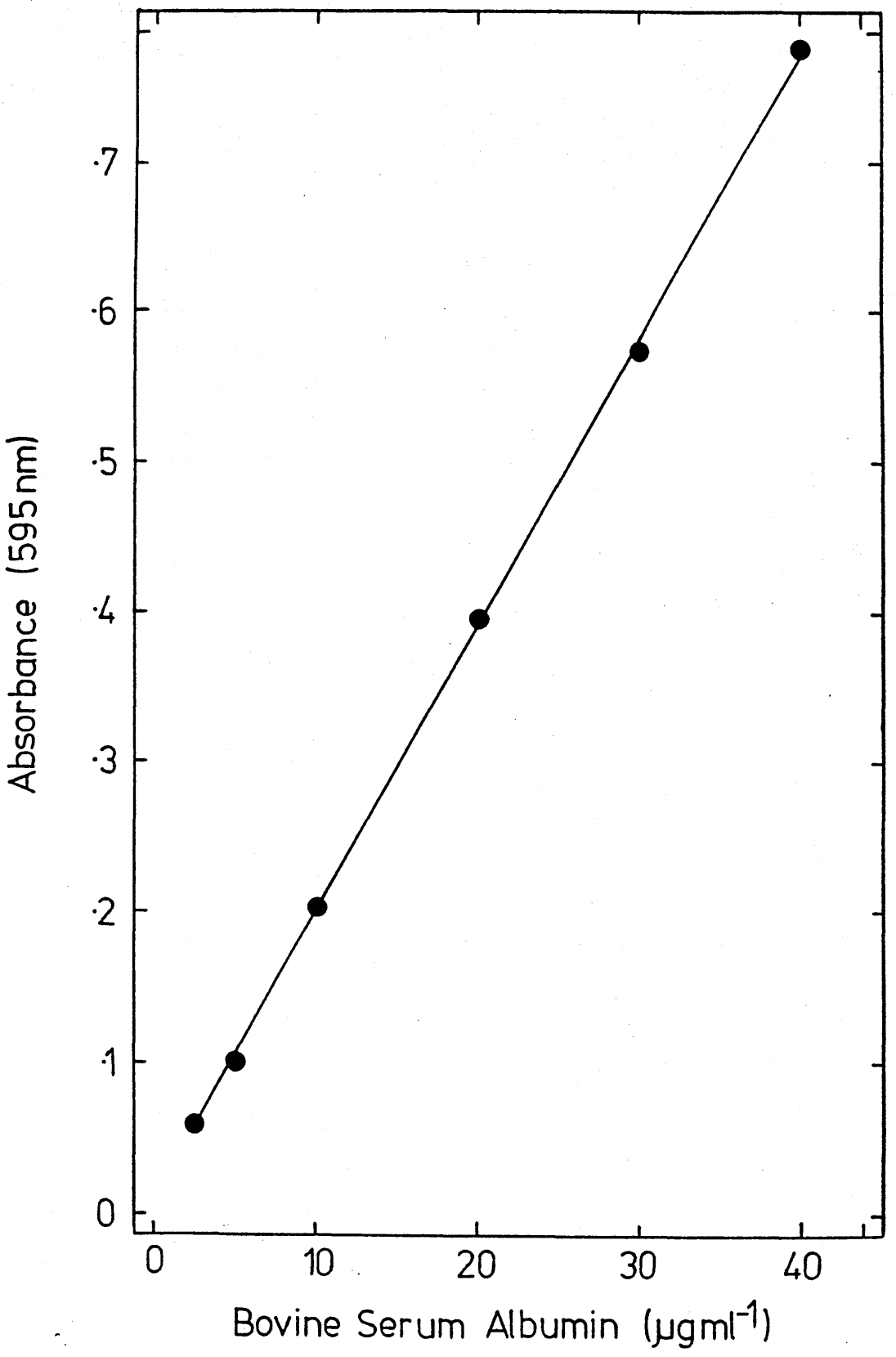

B
27
24
21
18
15
12
9
0
25
50
75
Frankia protein (µg ml⁻¹)
Time (secs)

Figure 3: Calibration curve for reaction of Bovine Serum Albumen with Pierce Protein Assay Reagent.


Absorbance (595nm)
·7
·6
·5
·4
·3
·2
·1
0
0
10
20
30
40
Bovine Serum Albumin (μgml⁻¹)

Preparation and quantification of crushed nodule inoculum

Nodule clusters, collected the previous day and stored overnight at 4°C, were first washed thoroughly to remove any soil. The nodule lobes were then separated and surface sterilised by agitating in 95% methanol for 5 minutes. Separated lobes were rinsed thoroughly in distilled water and the upper 2 to 3mm of the tips removed with a sterile razor. Three cm^3 of nodule tip material was homogenised using a Potter Elvehjem homogeniser and diluted to 100ml with distilled water. This homogenate was used to inoculate the seedlings.

Fruit germination, seedling preparation and inoculation

A.rubra fruits were collected in November 1983 from 2 trees growing in a pure stand at Lennox Forest. *A. rubra* fruits from McNab's Farm, Vancouver Island; Menzies Bay, British Columbia and Prince Rupert, British Columbia were kindly provided by Drs. M. Cannell and L. Sheppard. *A. glutinosa* fruits were collected from Milngavie in 1984. Fruits were sown thinly in seed trays containing Perlite moistened with a nutrient solution as in 2.1.4. Forty days after sowing, that is, after emergence of the first true leaf but before emergence of the second, the seedlings were transferred carefully to 1.25l capacity plastic growth bags (see 2.1.4 for supplier), filled previously with Perlite moistened as before and stood in groups of 6 within seed trays lined with a plastic bag. Three seedlings were transferred to each bag. Four days after transfer individual seedlings were inoculated by applying 1ml of the appropriate inoculum at the base of the shoot using a 1ml syringe fitted with a 21 guage needle. In batches 1 to 4 a

total of 54 *A. rubra* seedlings contained in 18 bags and 3 trays were inoculated with each inoculum. Plants in 12 bags were assigned to be used for growth analysis and those in the further 6 bags for the determination of the relative rates of hydrogen evolution, hydrogen uptake and acetylene reduction described in 2.2.5. Eighteen non-inoculated control seedlings in 6 bags were also set up for each inoculated group. In batch 5 a total of 36 *A. rubra* seedlings contained in 12 bags and 2 trays and 36 *A. glutinosa* seedlings in a further 12 bags were inoculated with each inoculum. Relative rates of hydrogen evolution and acetylene reduction were carried out on the remaining plants left after harvest as described in 2.2.5. Eighteen non-inoculated control seedlings in 6 bags were set up for each species. In Batch 6 24 *A.rubra* seedlings from each seed provenance and contained within 12 bags and 2 trays were inoculated with the appropriate inoculum. The trays were randomised within each inoculum group and maintained in a single growth cabinet. Eighteen non-inoculated control seedlings were set up for each provenance.

Growth conditions, plant maintenance and harvest

The plants were grown in 2 controlled environment growth cabinets (16 hours light, 21°C; 8 hours dark, 19°C). Light was provided by a mixture of daylight and warm-white fluorescent tubes which provided 76±4.2μmol m^{-2} s^{-1} light in one growth cabinet and 80±1.7μmol m^{-2} s^{-1} light in the other (n=4); measured using a Quantum Measuring System at seedling height. Batches 4, 5 and 6 were grown in one cabinet throughout (16 hours light, 21°C; 8 hours dark, 19°C) provided with 79±3.5μmol m^{-2} s^{-1} light by the same mixture of tubes. Bags were not

randomised between strains within the cabinets as this would have increased considerably the risk of strain cross-contamination. Small, measured quantities of nitrogen were given in the form of Liquinure to the inoculated seedlings and appropriate control seedlings when necessary 1 to 3 weeks after inoculation. Five seedlings were randomly selected at 7 day intervals and carefully examined for nodulation using a dissection microscope where necessary. In batches 1 to 3 at 14 day intervals throughout the growth period the plants were transferred to the alternate growth cabinet in order to compensate for any small environmental differences which may have existed between the 2 cabinets. Nutrients (100ml of full strength Crones-N and Hoagland's A-Z micronutrients, per bag) were applied 51 and 75 days after nodulation. Plants were watered daily with distilled water except during the first 7 days after inoculation.

96 days after nodulation of inoculated plants both inoculated and uninoculated control plants were harvested. Plants were inspected for nodulation and the percentage of plants nodulated calculated. Assessments were carried out on the tallest inoculated plant from each bag originally assigned for growth assessments; that is, 12 from each inoculated set and all the controls. Plant root, shoot and nodule dry weights were determined together with the number of nodules and nodule distribution. Ratios of root to shoot and plant to nodule dry weight were calculated. Dry weights were calculated after drying in an oven at 80°C to constant weight. The nitrogen content of harvested plants was determined using a semi-micro Kjeldahl procedure as described in 2.2.3. Analysis of hydrogen evolution, hydrogen uptake, and acetylene reduction was performed on the appropriate plants as described in 2.2.5.

Statistical analysis

Analysis of Variance was carried out on measured and calculated parameters of growth. Where significant ($p < 0.05$) effects of treatments were indicated using one factor Analysis of Variance and the significance of difference between means was determined using Duncan's Multiple Range Test (see 2.2.3 for details). Two factor Analysis of Variance was carried out where appropriate using the statistical package GENSTAT V (Lawes Agricultural Trust, Rothamsted Experimental Station, Release 4.04B) run on the University of Edinburgh EMAS computers.

2.2.5 Hydrogen metabolism of strains *in vivo*

Source of nodules and nodule preparation

Plants were inoculated and grown as described in 2.2.4. In all cases the assays described were carried out within 4 weeks of the 96 day harvest period.

Plants were removed from the controlled environment growth cabinets in which they had been maintained immediately prior to the commencement of the assay. Loose Perlite was removed by shaking followed by washing carefully in tap water. Care was taken throughout to minimise handling the nodules or otherwise damaging them since this has been demonstrated by Wheeler *et al.* (1978) to cause reductions in rates of

acetylene reduction. Small sections of root with the nodules still attached were cut from the plant. It is on these sections that the assays were performed.

Hydrogen evolution and hydrogen uptake assays were performed on nodules from different plants. Assays of acetylene reduction were performed on nodules immediately after the completion of hydrogen evolution or hydrogen uptake assays with the exception of time course assays where they were performed on nodules immediately after removal from the plant. Replicate assays (usually 3) were performed on nodules from different plants.

Hydrogen evolution assay

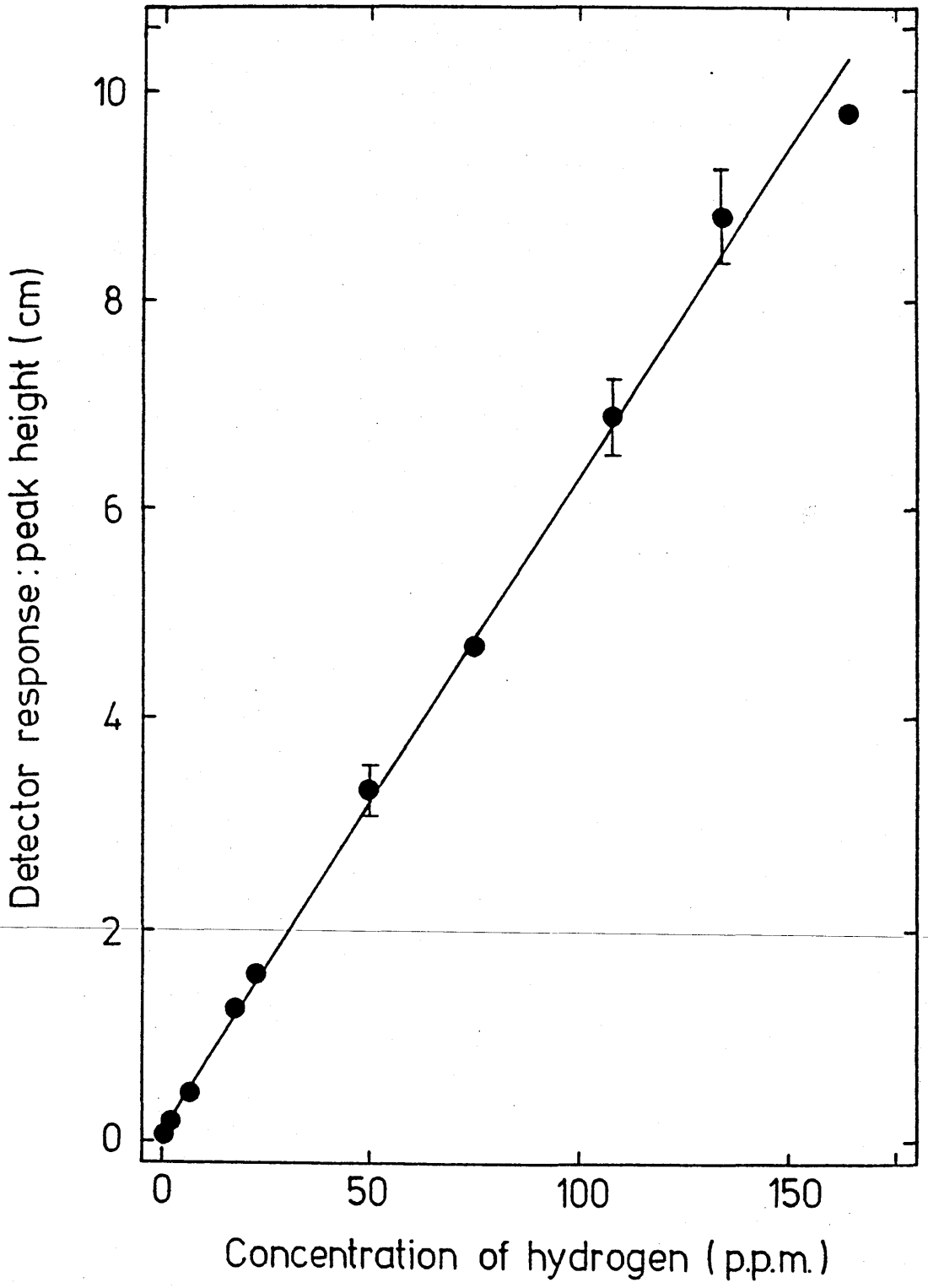
Approximately 0.5g fresh weight nodules together with associated root pieces were placed into a round bottomed flask of approximately 60ml volume together with a glass bead to aid mixing, sealed with a Subaseal (Subaseal Vaccine Stopper, Freeman Ltd.) and incubated in the dark at 20°C for 60 minutes. Duplicate 0.5ml samples of the gas phase were taken 0, 30, and 60 minutes after sealing using disposable 1ml syringes fitted with a needle (Yale, 25G). An exception to this was during the time course assays where the nodules were incubated for a total of 210 minutes and samples withdrawn after 0, 5, 10, 20, 30, 60, 120, 150, 180, 210 and 240 minutes. Assays of the hydrogen content of the samples were made using a portable gas chromatograph equiped with a Taquchi number 812 gas sensor and constructed as described by Holfeld *et al.*(1979). The chromatographic column was a 5 foot x ⅛ inches stainless steel column of Molecular Sieve 5A (60 - 80 mesh)

and the carrier gas air (flow rate 75ml min^{-1}). Preliminary experiments showed that the relationship between peak height and concentration was linear over the range required (Figure 4). Quantification of detector response was by comparison with the peak height given by a standard gas (109 p.p.m. hydrogen in nitrogen, British Oxygen Company Special Gases). Corrections were made for temperature, actual flask volume, sampling and nodule and root volume. In practice a fresh weight to volume conversion factor was calculated in preliminary experiments and this used throughout.

Hydrogen uptake assay

Approximately 0.5g fresh weight nodules together with associated root pieces were placed into a volumetric flask of approximately 130ml volume together with a glass bead and sealed with a Subaseal. One ml of the gas phase was then removed and replaced with 1ml of hydrogen using a disposable syringe fitted with a needle giving a gas phase of approximately 0.75% hydrogen. Flasks were incubated in the dark at 20°C for 60 minutes and 0.7ml samples were removed after 0, 30 and 60 minutes. An exception to this was during the time course assays where the nodules were incubated for a total of 210 minutes with samples withdrawn after 0, 30, 60, 90, 120, 150, 180 and 210 minutes. All samples were diluted by injection into flasks of approximately 60ml volume which had previously been sealed with a Subaseal and which contained a glass bead. This dilution was necessary in order to adjust the concentration of hydrogen in the sample to within the range over which the response of the detector was linear. Duplicate 0.5ml samples

Figure 4: Calibration curve for response to H_2 of Figaro gas sensor.
Figures are means of 4 replicates. S.E. < 0.2cm not shown.


Detector response: peak height (cm)
0
2
4
6
8
10
0
50
100
150
Concentration of hydrogen (p.p.m.)

of the gas phase were taken from these flasks and assays of the hydrogen content of the samples were made as in the section above. Corrections were made as in the previous assay with an additional correction for dilution.

Acetylene reduction assay

With the exception of the time course assays where the assays were performed on freshly harvested material this assay was performed after the completion of one of the above assays. In the case of the hydrogen evolution assay the Subaseal was removed and replaced allowing the pressure and composition of the gas phase to equilibrate with that of the laboratory before the assay was begun. In the case of those nodules on which a hydrogen uptake assay had been performed the nodules were first transferred carefully to a flask of approximately 60ml volume before resealing with a Subaseal. In all cases 5ml of the gas phase was removed and this was replaced with 5ml acetylene giving an acetylene concentration of about 8%. The flask was incubated in the dark at 20°C for 60 minutes and duplicate 0.5ml samples withdrawn at 0, 30, and 60 minutes. An exception to this was during the time course assays where the nodules were incubated for 210 minutes and samples were withdrawn after 0, 30, 60, 90, 120, 150, 180 and 210 minutes. Assays of ethylene produced were made using a Pye 104 Gas Chromatograph operated isothermally (60°C) with a 1 metre x ¼ inch steel column of Poropak N (100 - 120 mesh), a carrier gas of oxygen free nitrogen (flow rate 35 mls min^{-1}) and flame ionisation detector (hydrogen / air flame). The relationship between peak height and concentration is linear over the range required and quantification of

detector response was by comparison with the peak height given by a standard gas (95 p.p.m. ethylene in argon, British Oxygen Company Special Gases). Corrections were made as in the hydrogen evolution assay described previously.

2.3.0 Factors Affecting Potential For Field and Nursery Inoculation with Selected Strains

2.3.1 Nodulation of *A. rubra* in the field and the infective capacity of different soils

Field observations and soil collection

Nodulation of *A. rubra* was examined at a number of sites in Northern Britain on which experimental plantings had been made by the Forestry Commission. The root systems of a small number of trees (usually 3), selected randomly from within the plots, were exposed as much as possible and the extent and distribution of nodulation recorded. Assessments of tree performance were also made. Soil samples were dug with a spade from the upper 10cm of the soil horizon at each site, at a distance of about 20 to 25 metres from the plantation border. Samples were stored and characterised as described in 2.1.1.

Determination of infective capacity of soil samples

For each soil type four, 4 inch diameter pots were filled with a 1:1 mix of the soil and Perlite (standard grade). Approximately 0.5g *A. rubra* fruits, harvested from Lennox Forest and stored in a refrigerator prior to use, were spread evenly over two of the pots and approximately 0.5g *A. glutinosa* fruits harvested from Milngavie and stored in the same way were spread evenly over the other two pots. The fruits were covered with a 1 to 2mm layer of Perlite (superfine grade) to maintain a high humidity and encourage germination. Four control pots were set up for each species, containing Perlite alone (standard grade:superfine grade, 3:1) moistened with a nutrient solution as described in 2.1.5. Plants were maintained in a heated greenhouse (above 15°C , 16 hour photoperiod). After 22 weeks 10 seedlings were removed from each pot and examined for the presence of nodules.

The capacity of those soil samples, which themselves failed to nodulate test seedlings, to permit infection of test seedlings when *Frankia* was incorporated was examined as follows. A suspension of *Frankia* strain 1.2.5[Q](b) was prepared as described in 2.1.5 and watered equally over the surface of each of the pots. After a further 6 weeks the same procedure was repeated using *Frankia* strain 1.1.1[BuC], prepared as previously described. After a further 10 weeks 10 seedlings were removed from each pot and examined for the presence of nodules. The percentage nodulation of seedlings was determined for each soil / species combination.

2.3.2 Glasshouse experiments to investigate interactions between Frankia , host plant species, seed provenance and soil type

Soils were collected from sites in Scotland (see Table 2 for details of sites and soils), sieved to <1cm, mixed using a concrete mixer and stored in plastic bags (25kg of soil to each bag) prior to sterilisation by irradiation. Eleven days after collection soils were subjected to 3.5 to 4 megarads of radiation in order to eliminate indigenous Frankiae which may have been present in the soils. Soil pH was determined (1:5, soil:distilled water) and chemical analysis of the soil nutrients carried out by the Institute of Terrestrial Ecology, Analytical Section, Merlewood Research Station, Cumbria.

Crushed nodules were used as a source of Frankiae in this experiment. These originated from: 1) *A. rubra* at Lennox Forest, 2) *A. glutinosa* at Milngavie (see Table 1 for site details), 3) nodules from water culture *A. rubra* inoculated with crushed nodules, origin Corvallis (see 2.2.3 for details) and 4) nodules from water culture *A. rubra* inoculated with the strain ArI5 (see 2.2.4 for details). Nodule clusters, collected on the same day as inoculation were first washed thoroughly to remove any soil. The nodule lobes were then separated and surface sterilised by agitating in 95% methanol for 5 minutes. Separated lobes were rinsed thoroughly in distilled water and 1.7g nodule lobe material ground with distilled water in a mortar and pestle and diluted to 500ml. This homogenate was used to inoculate seedlings which were grown from *A. rubra* fruits collected from Terrace, British Columbia, (Latitude 49°9') and from Timberland Lake, Vancouver Island, (Latitude 54°31') and *A. glutinosa* fruits

collected from Loch Fyne, Scotland (Latitude 56°9'). This material was kindly provided by M. Cannell and L. Sheppard. Fruits from each of these sources (stratified previously) were sown thinly in seed trays containing Perlite moistened with a nutrient solution as in 2.1.4.

Table 2: Details of sites from which soils were collected for use in experiments in 2.3.2.

Site	Location[1]	Soil description	Soil pH[2]
Leadburn	247512	Brown earth	5.2
Leadburn	225565	Acid peat	3.8
Elibank	390370	Acid brown earth	4.4

[1] O.S. sheet 73, Peebles and Galashiels
[2] 1:5 distilled water.

Treatments were as follows: a) soil type (3) b) inoculum source (4) c) species or fruit provenance (3). Together with a non-inoculated treatment this makes a total of 3 x 5 x 3 ie 45 treatments. Treatments were replicated 20 times, a total therefore of 900 experimental units.

Thirty two days after sowing seedlings were inoculated with equal quantities of the appropriate inoculum by pouring the nodule homogenate equally over the surface of the appropriate seed trays. Immediately prior to inoculation and additionally after 44 days 0.1 and 0.3ml Tomorite fertiliser (Fisons, containing N, P and K) respectively was applied to each of the seed trays in 200ml water. Fifty eight days after sowing seedlings were transferred to 2 inch square pots, filled previously with the appropriate soil; 2 seedlings were transferred to each pot. After 107 days single seedlings (1 from each pot) were transplanted into 5 inch square pots, filled

previously with the appropriate soil and randomised within 5 split blocks, the 20 plants allocated 4 per block on the basis of height. The blocks were split by soil type to minimise shading effects but, within soil type, the 4 replicates were fully randomised. The height of each plant was measured at 170 days after which time most had set bud. At this time each plant was placed individually on an aluminium dish containing approximately ½ inch Perlite (standard grade) to prevent waterlogging. During the period of dormancy which followed extensive damage was caused to a number of plants by vine weevil (*Otiorrhyncus sulcatus*). The damaged plants were removed from the experiment and the remainder treated with Murphy's gamma BHC, followed by 2 drenches with malathion. After 1 month no living larvae could be found and very few beetles seen. Two hundred and ninety six days after sowing plant top growth on some soils showed symptoms of nutrient deficiencies and after 378 days (at the begining of the next growing season), therefore, 10ml of molar K_2HPO_4 was added to each pot, providing 0.3 and 0.78g of phosphate and potassium respectively. Throughout the germination and growth period pots were maintained in an unheated glasshouse at the Institute of Terrestrial Ecology, Bush Estate, Penicuik and watered regularly with tap water.

Plants were harvested 511 days after sowing. Plant height, root, shoot, and nodule dry weights were recorded and the number of nodules determined for each plant. Dry weights were calculated after drying in an oven at 80°C to constant weight. Stem material was combined from all the plants within individual blocks and root and nodule material from all plants within a single treatment. The nitrogen content of roots plus nodules was determined by a semi-micro Kjeldahl technique following a procedure described in 2.2.3. Determinations of total

shoot nitrogen were made at the Institute of Terrestrial Ecology, Analytical Section.

Statistical Analysis

Analysis of Variance was carried out where appropriate using the statistical package GENSTAT V (Lawes Agricultural Trust, Rothamsted Experimental Station, Release 4.04B) run on the University of Edinburgh EMAS computers.

2.3.3 Effects of aqueous soil extracts on the growth of *Frankia* strains

Determination of growth characteristics of experimental strains

Frankia strains used in the comparison were 1.2.19[Q] and 1.2.23[Q](b) (see 3.1.0 and 3.2.0 for details of isolation and characteristics). Strains, maintained in BuCT and subcultured 28 days previously, were first homogenised by passing five times through a 50ml syringe fitted with a needle (Yale, 21G). Protein estimations were performed on duplicate 2ml samples of the homogenate as described in 2.2.3 and the homogenate volume adjusted with BuCT medium to give a protein content of 13μg ml^{-1} for inoculation of experimental flasks.

Conical flasks (100ml) containing 40ml filter sterilised medium (Pharme-Aide Fluid Dispensing System fitted with a Millex GS, 0.2μm cartridge) were each inoculated with 1ml of the standard homogenate. The flasks were randomised within an incubator at 28°C. At regular intervals over a period of 40 days the contents of a number of flasks were collected on 20μm membrane filters (Whatman, 47mm) using a millipore apparatus *in vacuo*, made up to a suitable volume with distilled water and assayed for protein as described in 2.1.3 after sonication of either a 4ml sample for 60 seconds in a cooled sonication chamber (Figure 2) or a 15ml sample for 60 seconds in a cold shoulder cell (Life Science Laboratories Ltd). Preliminary experiments had shown that no further protein yield was obtained after sonication of samples for 50 and 45 seconds respectively (Figure 2). The pH of the culture medium was measured using a Howe Model 6030 pH meter.

Preparation of aqueous soil extracts

Soils were collected and stored as described in 2.3.1 from a number of Scottish sites. Extracts were prepared within 2 to 3 days of soil collection. The soil was first sieved to remove any particles of soil larger than 2mm. Four hundred ml distilled water was then added to 400ml of soil in a 1l borosilicate glass Duran bottle (Gallenkhamp) and shaken on a rotary shaker at 20°C for 17 hours. After allowing to stand the aqueous extract was decanted from the soil and centrifuged at 3000 r.p.m. for 1 hour (Mistral M.S.E. 21). The supernatant was then filtered sequentially *in vacuo* through Whatman 44 filter paper (9cm), Whatman GF/C (9cm) filter paper, Whatman 0.45μm membrane

filter (47mm) and finally Whatman 0.2µm membrane filter (47mm). Distilled water was passed through the same filters as a control and extracts were stored in a coldroom at 4°C overnight before inclusion in the appropriate medium.

Determination of the effects of soil extracts on *Frankia* growth

Media were prepared as for BuCT with the relevant soil extracts being substituted for distilled water in all cases but one. The complete medium was filter sterilised.

Conical flasks (100ml) containing 40ml of each medium were inoculated with aliquots of 13µg protein of 1.2.19[Q] or 1.2.23[Q](b) prepared as described earlier, randomised, sealed with Nescofilm and incubated in static culture in the dark at 28°C. After 25 days the contents of the flasks were harvested and yields determined as described above.

2.3.4 The growth of *Frankia* in aqueous soil extracts

A homogenate of strain 1.2.23[Q](b) was prepared as described in 2.3.3. Aliquots (1ml) were then added to a number of tubes (2cm x 15cm) to which 10ml of either the appropriate soil extract (prepared as described in 2.3.3), BuCT (to act as a comparitor) or a pH 6.8 buffer (1g l^{-1} K_2HPO_4 and 0.67g l^{-1} NaH_2PO_4) had been added. The

tubes were covered with aluminium caps (Oxoid) sealed with Nescofilm and maintained in static culture in the dark at 28°C.

After 52 days growth was assessed against the control tubes and scored as + or -. Sodium propionate was added (filter sterilised) to a number of the tubes in any treatment in which no growth was observed at a final concentration of 0.5g l^{-1}. After a further 52 days growth was again assessed as described previously.

2.3.5 Competition between strains for nodulation of soil-grown *A. rubra*

Preparation of inoculum

Two sources of inoculum were used. 1) Crushed nodules from Lennox Forest which normally produces spores in nodules (sp^+) and 2) ArI5 a non-indigenous *Frankia* from North America which is highly effective in association with *A. rubra* (see Dillon and Baker, 1982 and 2.2.4) and does not form spores in nodules (sp^-). The presence or absence of spores in the cells of nodules produced by infection with the respective strains was used as a morphological marker to study competition between the two strains.

A.rubra nodules were collected from Lennox Forest as described in 2.2.4. After washing thoroughly to remove soil the nodule lobes were separated and surface sterilised by agitating in 95% methanol for 5 minutes. Separated lobes were rinsed thoroughly in distilled water and

the upper 2 to 3mm of the tips removed with a sterile razor. The nodule lobe tips were then homogenised with distilled water (Sorval, 30,000rpm for 30 seconds). This homogenate was diluted with distilled water and used to inoculate the appropriate seedlings.

ArI5, maintained previously on Bu and sub-cultured into Bu approximately 8 weeks previously was separated from the medium by passing through a 0.20μm (Millipore, 47mm) membrane filter using a Millipore Apparatus *in vacuo* and the residue washed thoroughly with distilled water. The washed residue was homogenised with distilled water as described for lobe tips.

Fruit germination, seedling inoculation, maintenance and preliminary assessments

Fruits harvested from *A. rubra* growing in Lennox Forest and stored in a refrigerator prior to use were sown thinly in seed trays containing Perlite moistened as in 2.1.5. Forty days after sowing and before the emergence of the second true leaf the seedlings were transferred to plastic bags (see 2.1.5 for supplier) filled previously with Perlite moistened as before and stood in groups of 12 within seed trays lined with a plastic bag. Three seedlings were transferred to each bag. Four days after transfer the seedlings were inoculated by pouring equal quantities of the relevant inoculum homogenate over the Perlite surface. A total of 270 seedlings were inoculated with Lennox crushed nodule inoculum and 630 seedlings with ArI5. The seedlings were watered regularly with tap water and for the first 4 weeks after inoculation were all given small measured quantities of Liquinure.

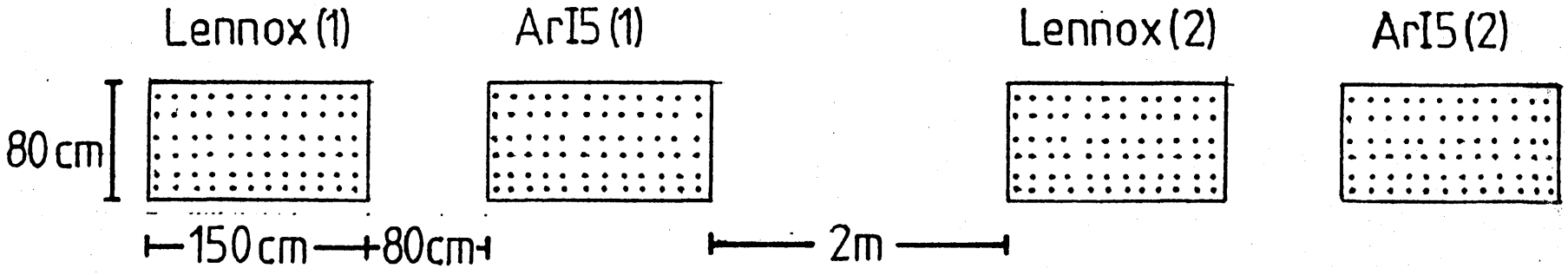
Plants were grown in a heated greenhouse (above 15°C, photoperiod 16 hours) with the photoperiod supplemented initially with 400W mercury vapour lamps. After 210 days 8 plants were selected randomly from each inoculated group. Plant height was measured and root, shoot and nodule dry weights determined after drying in an oven at 80°C to constant weight. The number of infection sites was also recorded. The nitrogen content of individual plants was determined using a semi-micro Kjeldahl technique following the procedure described in 2.2.3. A further 5 plants were selected randomly from each inoculated group and 2 nodule lobes examined as in 2.2.1 in order to determine spore production by *Frankia* in the nodules. A number of the remaining plants were selected randomly for planting in the plots as described below.

Preparation of plots, planting and maintenance

Soil was collected from the upper 15cm of the horizon from under a pure stand of *A. rubra* at Lennox Forest and mixed, to ensure homogeneity, before placing into 4 pits dug previously and measuring 150cm x 80cm x 30cm deep. The pits were contained within an unheated glasshouse under natural light conditions. Seventy two plants inoculated with each of the strains were planted out in June 1984 according to the design illustrated in Figure 5. The two plots containing plants inoculated with ArI5 were each watered with an ArI5 / distilled water homogenate, prepared as described previously, on planting. The plots were watered regularly with tap water.

Figure 5: Diagram of plot layout in competition experiment.

Dots within plots represent individual plants spaced at 0.1m.


Lennox (1)
ArI5 (1)
Lennox (2)
ArI5 (2)
80 cm
150 cm
80cm
2m

Plant harvest

In February 1986, after a further 20 months growth plants were harvested. Assessments were carried out on 3 plants, selected randomly from each plot. Five nodule clusters were selected randomly from each plant; nodules at the root crown were excluded. Duplicate lobes were examined as in 2.1.1. for the presence of spore cells.

2.3.6 Field tests of Selected *Frankia* strains

This experiment was carried out at the Forestry Commission's Nursery at the Bush Estate, Penicuik, following nursery practice as far as possible. A number of seed beds (3 x 1 metre) were prepared and sterilised with Basamid, a soil sterilant (Robertsons Agrochemicals, contains Dazomet which releases methyl isothiocyanate) at 380kg ha^{-1} in late autumn and rotavated in spring. Each bed was separated by a buffer zone 1 metre in width in which was set a plastic barrier to a depth of 30cm to prevent the spread of inoculum. Immediately prior to sowing the beds were fertilised with a basal fertiliser which contained phosphorus and potassium and in all cases but one, no nitrogen (NPK: Enmag, Scottish Agricultural Industries, to give NP and K at 58.5,114.0 and 108 kg ha^{-1} respectively and PK: mix to give P and K at 111.0 and 105 kg ha^{-1} respectively). During the first week of April the beds were sown with fruits of *A. rubra* obtained from Lennox Forest at a density of 2400 per plot.

Preparation of inoculum

The inocula used were *Frankia* 1.2.5[Q](b), ArI4 and crushed nodules obtained from Lennox Forest. Nodule clusters collected the previous day from a pure stand of *A. rubra* and stored overnight at 4°C were first washed thoroughly to remove any soil. The nodule lobes were then separated and surface sterilised by agitating in 95% methanol for 5 minutes. Separated lobes were rinsed thoroughly in distilled water and the upper 2 to 3mm of the tips removed with a sterile razor. Twenty five grams of nodule tip material was homogenised in tap water using a Sorval Homogeniser operated at full speed for 30 seconds and the homogenate diluted to 10 litres in a watering can. This suspension was used to inoculate the seedlings.

Strains were maintained previously on BuCT medium and were subcultured into 1 and 2 l flasks some 8 weeks prior to use. Cells were harvested from the medium by filtration through a 20µm nylon mesh. The retained mycelium was rinsed thoroughly with distilled water, homogenised as described previously for the nodule material and diluted to 10 l in a watering can. This suspension was used to inoculate the seedlings.

Seedling inoculation, plant maintenance and harvest

There were 5 treatments in total (see Figure 6 for layout).

1) basal fertiliser containing nitrogen (NPK),

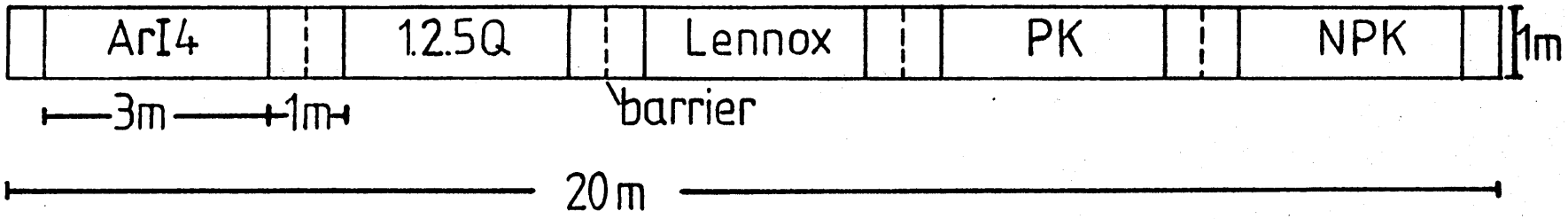
2) basal fertiliser containing no nitrogen (PK),

3) PK and inoculation with crushed nodules,

4) PK and inoculation with 1.2.5[Q](b),

5) PK and inoculation with ArI4.

Figure 6: Diagram of plot layout in nursery experiment.


ArI4
1.2.5Q
Lennox
PK
NPK
1m
3m
1m
barrier
20 m

Where appropriate, inoculum was watered onto the beds 4 weeks after sowing. Immediately after inoculation the plants were watered. In the case of 1.2.5[Q](b) and ArI4 inoculation was repeated after a further 4 weeks, due to a sustained period of dry weather which it was considered may have reduced the viability of the endophyte. This was suggested by the chlorotic appearance of the seedlings compared to those inoculated with crushed nodule inoculum. The plots were covered with a nylon mesh to prevent damage to the plots or transfer of endophyte from one plot to another by birds and watered regularly with tap water. There were no further fertiliser treatments.

Inoculated and uninoculated plants were harvested 187 days after sowing. The following assessments were carried out on each of 20 plants randomly selected from each treatment; plant height; root, shoot, and nodule dry weights; and the number of infection sites. Dry weights were calculated after drying in an oven at 80°C to constant weight. Analyses of total nitrogen content were carried out as described in 2.2.3.

Statistical analysis

Analysis of Variance was carried out on plant dry weight measurements using the statistical package MINITAB. Where significant ($p < 0.05$) effects of treatments were indicated using this technique the significance of the difference between means was determined using Duncan's Multiple Range Test (see 2.2.3 for details of both techniques).

3.0.0 Results

3.1.0 Isolation of *Frankia*

Many different methods have been used by other workers for the isolation of *Frankia*. The effectiveness of 3 of the most commonly used of these for the isolation of *Frankia* from root nodules of *A. glutinosa* from a number of different sources is shown in Tables 3 to 5.

None of the methods was successful in the isolation of the endophyte from field-collected nodules (stored at -20°C) from either Hoogmade or Balmaha (Table 3); nodules from both sites were identified as being sp^+. In attempts from field-collected nodules from Diemen (sp^-), nodules (also stored at -20°C) however, endophyte growth was observed using each of the 3 methods. The largest numbers of colonies on isolation plates were obtained using the sucrose density fractionation method. With each method growth was on the same medium and inclusion of a total lipid extract (TL) was obligatory. Using the differential filtration method the inclusion of propionate increased considerably the number of colonies visible after 25 days. With both the sucrose density fractionation method and the nodule fragment method, however, the addition of propionate had little or no observable effect on total number of colonies or percentage of fragments with hyphal outgrowths; although hyphal outgrowth from the nodule fragments appeared to be delayed it is possible that some were overlooked because of difficulties in the detection of these

outgrowths under the microscope. When an isolation was attempted using the differential filtration technique from nodules harvested from water culture grown plants previously inoculated with Diemen (sp^-) nodules, however, again TL was obligatory but in this case inclusion of propionate clearly inhibited growth (Table 4). It seems, therefore, that both method of isolation and the method of plant culture can have significant effects on the nutritional demands of *Frankia* on isolation.

Where isolation of the endophyte was attempted from nodules obtained by the inoculation of water culture plants with *A. glutinosa* crushed nodules from Balmaha, again there were differences dependant upon the method and media used. Both the differential filtration and the nodule fragment methods were successful, with the sucrose density fractionation method producing no hyphal outgrowths or colonies at all (Table 5). Of the two successful methods the differential filtration method was overwhelmingly more successful and the media required neither propionate nor TL in order to support growth. Using the nodule fragment method, however, growth occurred from only a small percentage of fragments and although limited growth did occur when fragments were incubated in medium containing propionate with or without TL, when fragments were incubated in medium without propionate only that medium to which no TL had been added would support growth. In the differential filtration method, however, the inclusion of TL in the medium always stimulated early growth so the significance of this one result obtained with the nodule fragment technique must be viewed with caution. These results generally reinforce the view that TL is stimulatory for *Frankia* growth during isolation.

Table 3: Isolation of *Frankia* from field collected root nodules of *A. glutinosa* (stored at -20°C for 2 days) using a number of different isolation techniques and media.

(A) Technique: Differential Filtration

Source of nodules	Diemen		Hoagmade		Balmaha	
Spore type	-		+		+	
Days after isolation	13	25	13	25	13	25
Medium[1]	%[2]	No[3]	%	No	%	No
M-N/Cas	0	0	0	0	0	0
M-N/Cas/TL	1.8±2.2	3.7±7.0	0	0	0	0
P-N/Cas	0	0	0	0	0	0
P-N/Cas/TL	2.2±0.5	43±6.8	0	0	0	0

(B) Technique: Sucrose Density Fractionation

Source of nodules	Diemen		Hoagmade		Balmaha	
Spore type	-		+		+	
Days after inoculation	13	25	13	25	13	25
Medium	%	No	%	No	%	No
M-N/Cas	0	0	0	0	0	0
M-N/Cas/TL	18±5.2	263±60	0	0	0	0
P-N/Cas	0	0	0	0	0	0
P-N/Cas/TL	17±7.0	296±65	0	0	0	0

(C) Technique: Nodule Fragments.

Source of nodules	Diemen			Hoagmade			Balmaha		
Spore type	-			+			+		
Days after inoculation	5	9	35	5	9	35	5	9	35
Medium	% fragments with hyphal outgrowth[4]								
M-N/Cas	0	0	0	0	0	0	0	0	0
M-N/Cas/TL	31	72	100	0	0	0	0	0	0
P-N/Cas	0	0	0	0	0	0	0	0	0
P-N/Cas/TL	0	47	100	0	0	0	0	0	0

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[1] Medium composition as described in Appendix 1.
[2] Percentage of vesicle clusters which show hyphal outgrowth. Figure shown is the mean of 6 replicate plates ± S.E.
[3] Number of macroscopically visible colonies per plate. Figure shown is the mean of 6 replicate plates ± S.E.
[4] 6 fragments per plate, figure shown is the mean of 6 replicate plates ± S.E.

Table 4: Isolation of *Frankia* from root nodules of water culture-grown A.glutinosa, previously inoculated with sp⁻ nodules from Diemen, using the Differential Filtration isolation technique.

Days after isolation	9	25
Medium.[1]	%[2]	No.[3]
M-N/Cas	0	0
M-N/Cas/TL	35±4.2	1093±201
P-N/Cas	0	0
P-N/Cas/TL	15±5.7	287±95

[1] Medium composition as described in Appendix 1.
[2] Percentage of vesicle clusters which show hyphal outgrowth. Figure shown is the mean of 5 replicate plates ± S.E.
[3] Number of macroscopically visible colonies per plate. Figure shown is the mean of 5 replicate plates ± S.E.

Table 5: Isolation of *Frankia* from root nodules of water culture grown *A. glutinosa* previously inoculated with sp$^+$ nodules from Balmaha using a number of different isolation techniques and media.

(A) Technique: Differential filtration.

Days after isolation	7	11
Medium[1].	% of vesicle clusters which show hyphal outgrowth[2] ± S.E.	
M-N/Cas	4±1.3	7±1.2
M-N/Cas/TL	18±6.3	22±4.5
P-N/Cas	10±4.0	25±8.1
P-N/Cas/TL	20±5.9	26±3.7

(B) Technique: Sucrose Fractionation.

Days after isolation	7	11
Medium.	% of vesicle clusters which show hyphal outgrowth ± S.E.	
M-N/Cas	0	0
M-N/Cas/TL	0	0
P-N/Cas	0	0
P-N/Cas/TL	0	0

(C) Technique: Nodule Fragments.

Days after isolation	7	11
Medium.	% nodule fragments with hyphal outgrowth.[3]	
M-N/Cas	2.7	2.7
M-N/Cas/TL	0	0
P-N/Cas	2.7	2.7
P-N/Cas/TL	5.6	5.6

---

[1] Medium composition as described in Appendix 1.

[2] Figure shown is the mean of 6 replicates ± S.E.

[3] Six fragments per plate, figure shown is the mean of 6 replicate plates ± S.E.

The effectiveness of the differential filtration method was compared further with a microdissection method using nodules collected from actinorhizal plants growing in Britain, Northern Europe and Northern America (Table 1). A number of different media were used with each method but attempts at isolation with the microdissection method were unsuccessful, even after incubation periods in excess of one year, and no putative *Frankia* isolates were obtained (Table 6). Of the microorganisms that were isolated few resembled actinomycetes morphologically and most were fungi. The differential filtration method, however, proved successful and a number of putative *Frankia* isolates were obtained with nodules from both sp^+ and sp^- sites (Table 7).

Colonies were normally visible macroscopically after 2 to 5 weeks although in the isolation of 1.1.11[P] colony development took 6 months. In general, most colonies were found on plates inoculated with the residue retained on the 20μm mesh with the greatest number of colonies appearing generally on plates inoculated with the greatest number of vesicle clusters. Light microscopy showed that the 20μm residue consisted mainly of vesicle clusters with little other nodule material; on isolation plates hyphae could be seen to grow from the vesicles. The 50μm mesh residue contained many of the larger nodule pieces and colonies were often difficult to distinguish from each other on isolation plates as there were often several separate outgrowths from a single nodule piece.

There were a number of differences between isolates obtained from different sites and sometimes from the same site. It can be seen from

Table 6: *Frankia* isolation using a microdissection method.

Site and Species[1]	Isolation Number &(spore nature of nodules)[2]	Isolation Medium[3]
A. glutinosa		
Milngavie	1(+)	Bu
	2(-)	P(+)
Tentsmuir	3(-)	Bu
A. rubra		
Lennox	1(-)	Bu Qmod
	2(+)	Bu Qmod
	3(-)	Bu
	4(-)	P(+)
McNab's Farm	5(-)	Bu

No *Frankia* strains were isolated using this method.

1 Site description Table 1.
2 For details of spore determination see 2.1.1.
3 Media details Appendix 1.

Table 7: *Frankia* isolation using a differential filtration method

SPECIES: *A. glutinosa.*

Site[1]	Isolation number[2]	Isolation medium[3]	Isolate designation[4]	No. of vesicle clusters plated x 10^4 and (mean no. colonies/plate[5])			Colony form[6]
				50µm	20µm	10µm	
Milngavie	1(ND)	Bu(cas)	1,1,1[BuC]	ND(ND)	ND(ND)	ND(ND)	Regular
	2(-)	QMOD	1,1,2[Q]	0,8(4)	1(0)	1(2)	Regular
	4(-)	Bu	1,1,4[Bu]	0,8(51)	1(95)	0,5(1)	Regular
		FMC+P	1,1,4[F]	0,8(9)	1(38)	0,5(0)	Regular
	5(+/-)	FMP+C	1,1,5[F]	0,1(240)	1(0)	1(82)	Regular
		Bu	1,1,5[Bu]	0,1(16)	1(12)	1(10)	Regular
	6(-)	P+E	-	2(-)	3(-)	1(-)	-
		QMOD	1,1,6[Q]	3(0)	3(4)	1(0)	Regular
Southport	3(-)	FMC+P	-	1(-)	1(-)	1(-)	-
		QMOD	-	0,5(-)	1(-)	1(-)	-
Tentsmuir	7(-)	Bu	1,1,7[Bu]	4(0)	2(0)	2(0)	Compact
		FMC+P	1,1,7[F]	4(64)	2(35)	2(15)	Compact
	11(-)	P(-)	1,1,11[P-]	ND(0)	ND(0)	ND(0)	Regular
		P(+)	-	ND(-)	ND(-)	ND(-)	-
		QMOD	-	ND(-)	ND(-)	ND(-)	-
Balmaha	8(-)	Bu	1,1,8[Bu]	0,5(28)	1(24)	0,5(21)	Regular
		QMOD	-	0,5(-)	0,8(-)	0,5(-)	-

Table 7 continued:

SPECIES: *A. glutinosa.*

Site[1]	Isolation number[2]	Isolation medium[3]	Isolate designation[4]	No. of vesicle clusters plated x 10^4 and (mean no. colonies/plate[5])			Colony form[6]
				50µm	20µm	10µm	
Balmaha	10(-)	Bu	1,1,10[Bu]	0,3(ND)	0,4(ND)	β	Regular
		FMC+P	-	0,3(-)	0,4(-)	β	-
		QMOD	-	0,3(-)	0,4(-)	β	-
		P+E	-	0,3(-)	0,5(-)	β	-
Bush	9(+/-)	QMOD	c	α(c)	α(c)	β	c
		P(+)	c	α(c)	α(c)	β	c
Rannoch Moor	12(ND)	Bu	-	α(-)	α(-)	β	-
		QMOD	-	α(-)	α(-)	β	-
	3(ND)	Bu	-	α(-)	α(-)	β	-
		QMOD	-	α(-)	α(-)	β	-
		P(+)	-	α(-)	α(-)	β	-
		FMC+P	-	α(-)	α(-)	β	-
Rumster 9	14(-)[7]	Bu	1,1,14[Bu]	0,1(140)	1(1400)	β	Diffuse
		QMOD	1,1,14[Q]	α(0)	1(900)	β	Diffuse

Table 7 continued:

SPECIES: *A. rubra*

Site[1]	Isolation number[2]	Isolation medium[3]	Isolate designation[4]	No. of vesicle clusters plated$\times 10^4$ and (mean no. colonies/plate[5])			Colony form[6]
				50µm	20µm	10µm	
McNab's Farm BC.	3(-)	Bu	1,2,3[Bu]	2(0)	2(0)	1(0)	Regular
		QMOD	-	2(-)	2(-)	0,8(-)	-
		FB	-	1,3(-)	2(-)	1(-)	-
Lennox	4(-)	Bu	1,2,4[Bu]	3(29)	4(21)	4(38)	Regular
		FMC+P	-	3(-)	4(-)	4(-)	-
	5(+)	P(+)	1,2,5[P+]	1(86)	2(98)	1(7)	Diffuse
		QMOD	1,2,5[Q](a) 1,2,5[Q](b)	1(55)	2(90)	2,5(4)	Compact Diffuse
	6(-)	Bu	-	α(-)	α(-)	-	-
		QMOD	-	α(-)	α(-)	-	-
	7(-)	P(+)	c	ND(c)	ND(c)	ND(c)	c
		FMC+P	c	ND(c)	ND(c)	ND(c)	c
Penicuik	9(ND)	P	-	0,3(-)	1(-)	α(-)	-
		QMOD	-	α(-)	0,8(-)	α(-)	-
Juneau, Alaska	10(-)	Bu	1,2,10[Bu]	0,8(0)	0,1(0)	0,5(0)	Compact
		FMC+P	-	1(-)	0,5(-)	0,1(-)	-

Table 7 continued;

SPECIES: *A. rubra*

Site[1]	Isolation number[2]	Isolation medium[3]	Isolate designation[4]	No. of vesicle clusters plated$x10^4$ and (mean no. colonies/plate[5])			Colony form[6]
				50µm	20µm	10µm	
Bush	8(+/-)	P(-)	c	α(c)	α(c)	β	c
		QMOD	c	α(c)	α(c)	β	c
	22(+/-)	Bu	1,2,22[Bu]	α(5)	1(89)	-	Regular
		QMOD	-	α(-)	1(-)	-	-
Shin 93, s.p. Western Centralia	11(-)[7]	Bu	-	α(-)	2(-)	-	-
		QMOD	-	α(-)	α(-)	-	-
s.p. Lennox	12(-)[7]	Bu	c	α(c)	α(c)	β	c
		QMOD	c	α(c)	α(c)	β	c
s.p.Big Qualicum River	13(-)[7]	Bu	1,2,13[Bu]	α(47)	0,3(233)	β	Diffuse
		QMOD	1,2,13[Q]	α(0)	0,3(1208)	β	Diffuse
Shin 65	14(-)[7]	Bu	-	α(-)	1(-)	β	-
		QMOD	-	α(-)	1(-)	β	-
Rumster 9	15(-)[7]	Bu	1,2,15[Bu]	0,1(29)	0,5(484)	β	Regular
		QMOD	1,2,15[Q]	0,1(0)	0,5(53)	β	Regular
Shin 45	16(-)	Bu	-	0,1(-)	1(-)	β	-
		QMOD	-	0,1(-)	19(-)	β	-

Table 7 continued;

SPECIES: *A. rubra*

Site[1]	Isolation number[2]	Isolation medium[3]	Isolate designation[4]	No. of vesicle clusters platedx10^4 and (mean no. colonies/plate[5])			Colony form[6]
				50µm	20µm	10µm	
Wauchope 9	17(-)	Bu	c	ND(c)	ND(c)	β	c
		QMDD	c	ND(c)	ND(c)	β	c
	23(-)	Bu	1,2,23[Bu]	α(0)	0,8(32)	β	Regular
		QMDD	1,2,23[Q](a) 1,2,23[Q](B)	α(24)	0,8(197)	β	Regular Diffuse
Falstone 6	18(+)	Bu	1,2,18[Bu](a) 1,2,18[Bu](b)	ND(10)	ND(197)	β	Compact Regular
		QMDD	-	ND(-)	ND(-)	β	-
South Yorks 9	19(-)	Bu	1,2,19[Bu]	ND(46)	ND(154)	β	Regular
		QMDD	1,2,19[Q]	ND(10)	ND(249)	β	Diffuse
Wykeham 116	20(-)	Bu	1,2,20[Bu]	ND(105)	ND(1194)	β	Diffuse
		QMDD	1,2,20[Q]	ND(221)	ND(1688)	β	Diffuse
Tumble	21(-)	Bu	-	0,1(-)	1(-)	β	-
		QMDD	-	0,1(-)	1(-)	β	-

Table 7 continued:

Site[1]	Isolation number[2]	Isolation medium[3]	Isolate designation[4]	No. of vesicle clusters platedx10⁴ and (mean no. colonies/plate[5])			Colony form[6]
				50µm	20µm	10µm	
SPECIES: *A. sinuata*							
Shin 45	1(-)	Bu	-	α(-)	0,1(-)	β	-
		QMOD	-	α(-)	0,1(-)	β	-
SPECIES: *A. incana*							
Woodhall	1(-)	Bu	-	0,1(-)	0,1(-)	β	-
		QMOD	-	0,1(-)	0,1(-)	β	-
Umea,	2(+)	Bu	1,4,2[Bu]	ND(ND)	ND(ND)	β	#
		BuCT	1,4,2[BuCT]	ND(ND)	ND(ND)	β	#
		QMOD	1,4,2[Q]	ND(ND)	ND(ND)	β	#
		P+	1,4,2[P+]	ND(ND)	ND(ND)	β	#
SPECIES: *Hippophae rhamnoides*							
Southport	1(-)	Bu	2,1,1[Bu]	0,2(0)	0,3(1)	β	Compact
		QMOD	-	0,2(0)	0,2(0)	β	-
Gullane	2(-)	FMC+P	2,1,2[F]	α(0)	1(0)	β	Compact
		QMOD	c	α(c)	α(c)	β	c

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1 See Table 1 for details,
2 Indicates isolation number for each species and (spore nature of nodules),
3 See Appendix 1 for details of media composition,
4 See Appendix 2 for details of nomenclature,
5 At 8 weeks,
6 Illustration of different colony forms Plates 2 to 4,
7 Not possible to observe spore cells, However, nodules were extremely small and poorly developed,
α Not possible to observe any vesicle clusters,
β Not used in this isolation,
c All plates removed due to contamination,
ND Not determined,

Table 8, for example, that isolation medium preferences existed and that, in some cases, colonies would only develop when residues were plated onto a particular medium. In all these cases, however, colonies developed on Bu medium when it was included. In fact isolates were obtained on Bu medium in every successful isolation attempt using the differential filtration method.

There were also clear morphological differences between different colonies on isolation plates. Hyphal sizes differed between different isolates (Table 9) in those cases where they were measured and ranged from 1.3 µm in 1.1.2[Q] to 2.8 µm in 1.2.4[Bu]. It was possible to distinguish 3 distinct colony forms regular, compact and diffuse (Plates 2 to 4). When strains were inoculated into the same medium (BuCT), although in some cases the colony form was unchanged in many it changed completely (Table 10). Given the relative instability or medium dependence of this characteristic it is unlikely to be useful in *Frankia* classification or identification. All isolates produced sporangia although in a number of cases they were small and poorly developed; however, this often varied greatly between colonies on the same plate (Plates 5 and 6). In liquid BuCT culture medium, however, sporangia developed on all isolates and contained many spores. Pigmentation of colonies was absent or slight and the majority of isolates appeared white on solid media. Pigmentation was either yellow, orange or pink and although sometimes limited to the colony was often excreted as a water-soluble pigment. On transfer to the liquid cultivation medium, BuCT, most isolates grew relatively well although there were large differences and some failed

Table 8: *Frankia* colony development on isolation plates inoculated with fractionated homogenates of *A. rubra* and *A. glutinosa* nodules.

Site and species	Isolation number	Medium				
		FMC+P	QMOD	P		Bu
				+	−	
A. glutinosa						
Milngavie	6	ND	+	−	ND	ND
Tentsmuir	11	ND	−	−	+	ND
Balmaha	10	−	−	−	ND	+
A. rubra						
Lennox	4	−	ND	ND	ND	+
Alaska	10	−	ND	ND	ND	+
Bush	22	ND	−	ND	ND	+
Falstone	6	ND	−	ND	ND	+
H. rhamnoides						
Southport	1	ND	−	ND	ND	+

+ Indicates development of colonies.
− Indicates no colony development.

Table 9: Hyphal diameter of some isolates.

Isolate	Hyphal diameter (μm)±S.E.
1.1.4[Bu]	1.4 ± 0.26
1.1.2[Q]	1.3 ± 0.19
1.1.4[F]	1.4 ± 0.15
1.1.7[F]	2.2 ± 0.26
1.1.7[Bu]	1.9 ± 0.19
1.1.8[Bu]	1.7 ± 0.26
1.2.4[Bu]	2.8 ± 0.23
1.2.5[Q](a)	2.5 ± 0.10
1.2.5[Q](b)	1.9 ± 0.30
1.2.5[P^+]	2.5 ± 0.30

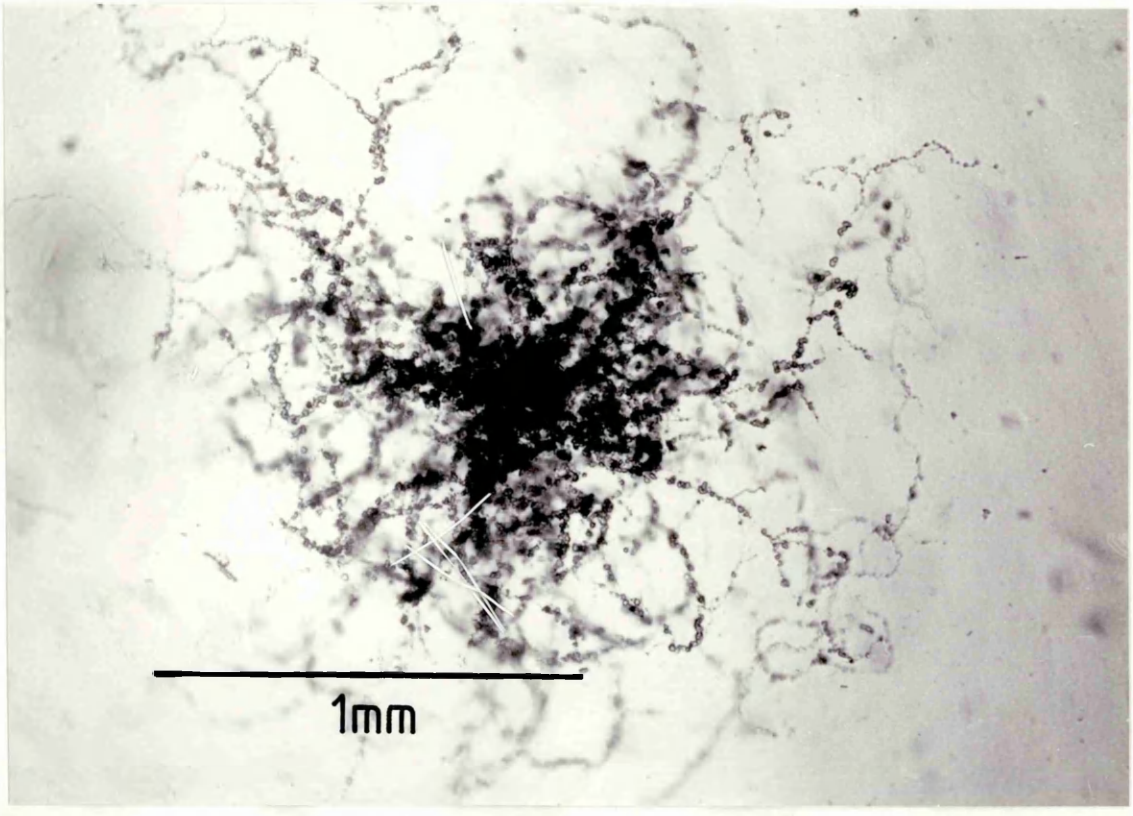
Figures are means of 20 replicate measurements.

Table 10: Observations on strains inoculated into agar-solidified media in petri dishes.

Strain	Isolation medium	Colony form on isolation	Colony form on BuCT
1.1.1[BuC]	BuC	Regular	Compact
1.1.2[Q]	QMOD	Regular	Compact
1.1.4[Bu]	Bu	Regular	Compact
1.1.4[F]	FMC+P	Regular	Compact
1.1.5[F]	FMC+P	Regular	Compact
1.1.5[Bu]	Bu	Regular	Compact
1.1.7[Bu]	Bu	Compact	Compact
1.1.7[F]	FMC+P	Compact	Compact
1.1.8[Bu]	Bu	Regular	Diffuse
1.1.14[Bu]	Bu	Diffuse	Diffuse
1.1.14[Q]	QMOD	Diffuse	Diffuse
1.2.3[Bu]	Bu	Regular	Regular
1.2.4[Bu]	Bu	Regular	Regular
1.2.5[P+]	P+	Diffuse	Diffuse
1.2.5[Q] (b)	QMOD	Diffuse	Diffuse
1.2.13[Bu]	Bu	Diffuse	Diffuse
1.2.13[Q]	QMOD	Diffuse	Diffuse
1.2.15[Bu]	Bu	Regular	Compact
1.2.23[Bu]	Bu	Regular	Regular
1.2.23[Q] (b)	Qmod	Diffuse	Diffuse
1.2.19[Bu]	Bu	Regular	Diffuse
1.2.19[Q]	QMOD	Diffuse	Compact
1.2.20[Bu]	Bu	Diffuse	Diffuse
1.2.20[Q]	QMOD	Diffuse	Diffuse

Plate 2: Regular colony form illustrated by 1.1.4[Bu] growing on Bu medium on isolation.

Plate 3: Compact colony form illustrated by 1.2.5[Q](a) growing on Qmod medium on isolation


1mm

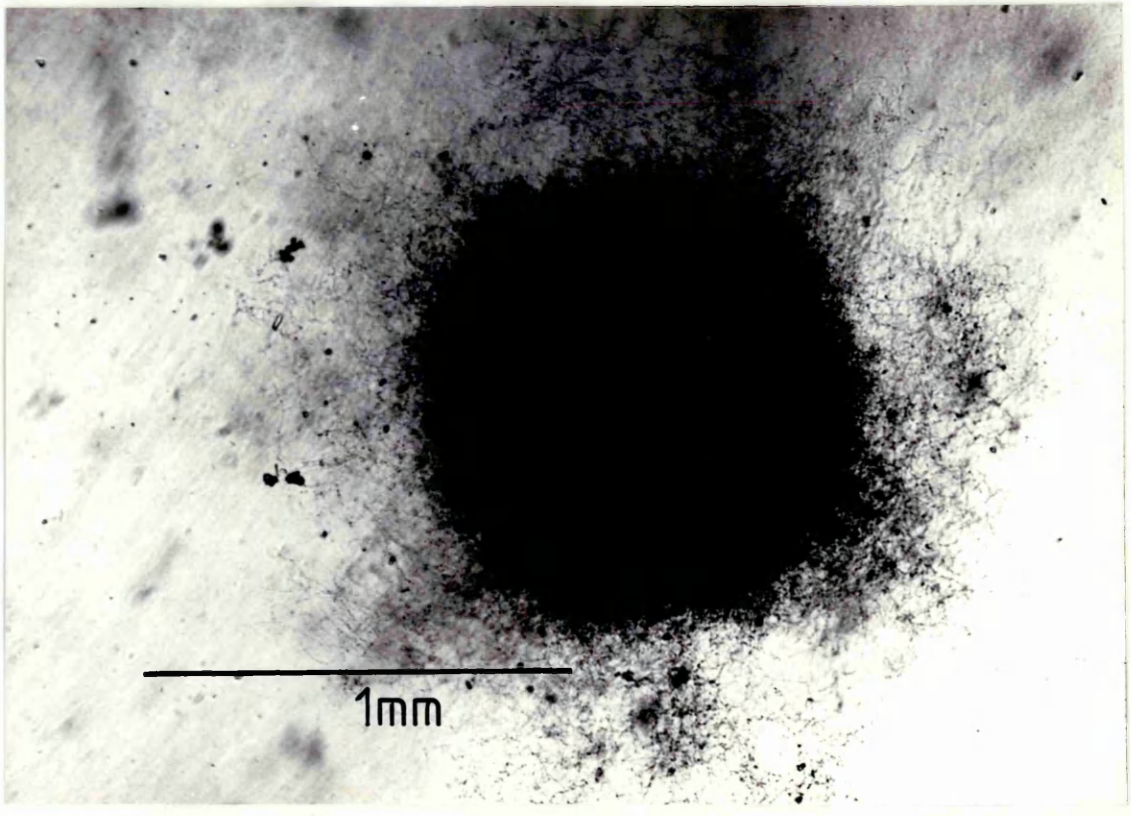
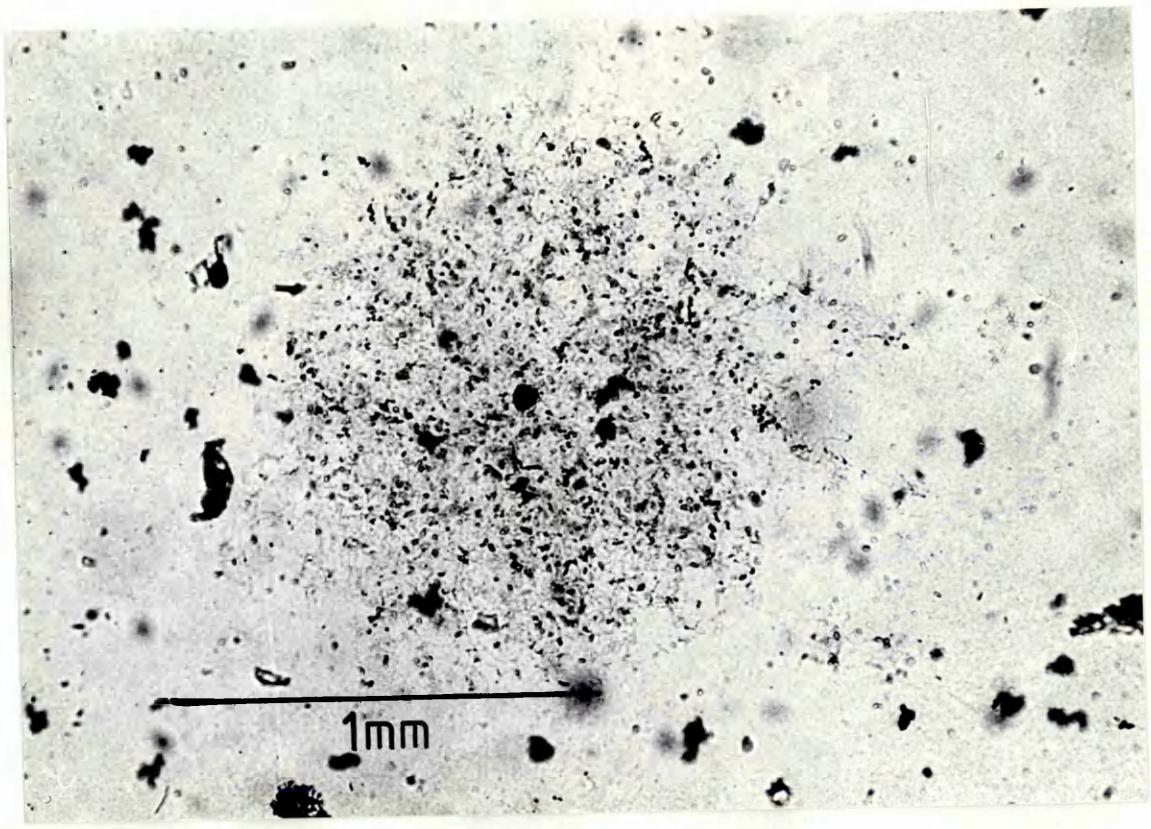

1mm

Plate 4: Diffuse colony form illustrated by 1.2.5[Q](b) growing on Qmod medium on isolation.

Plate 5: Heavily sporulating colony of 1.1.4[Bu] growing on Bu medium on isolation.


1mm

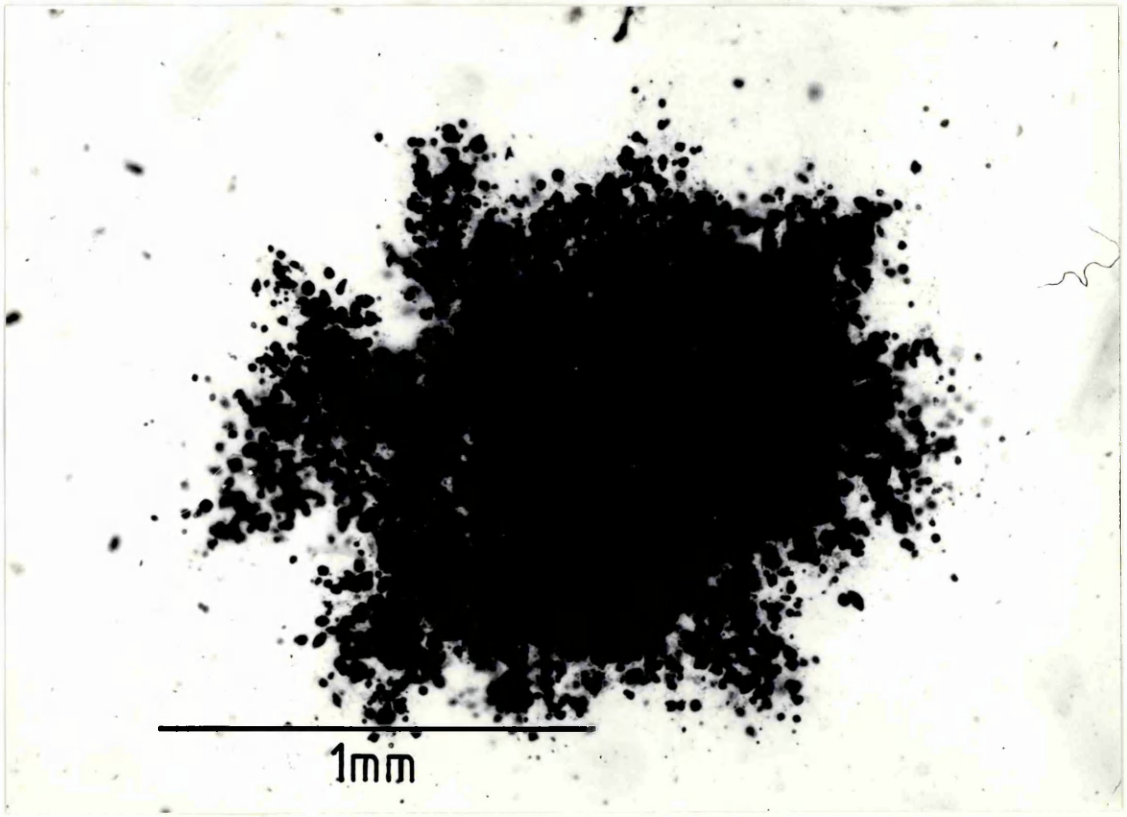
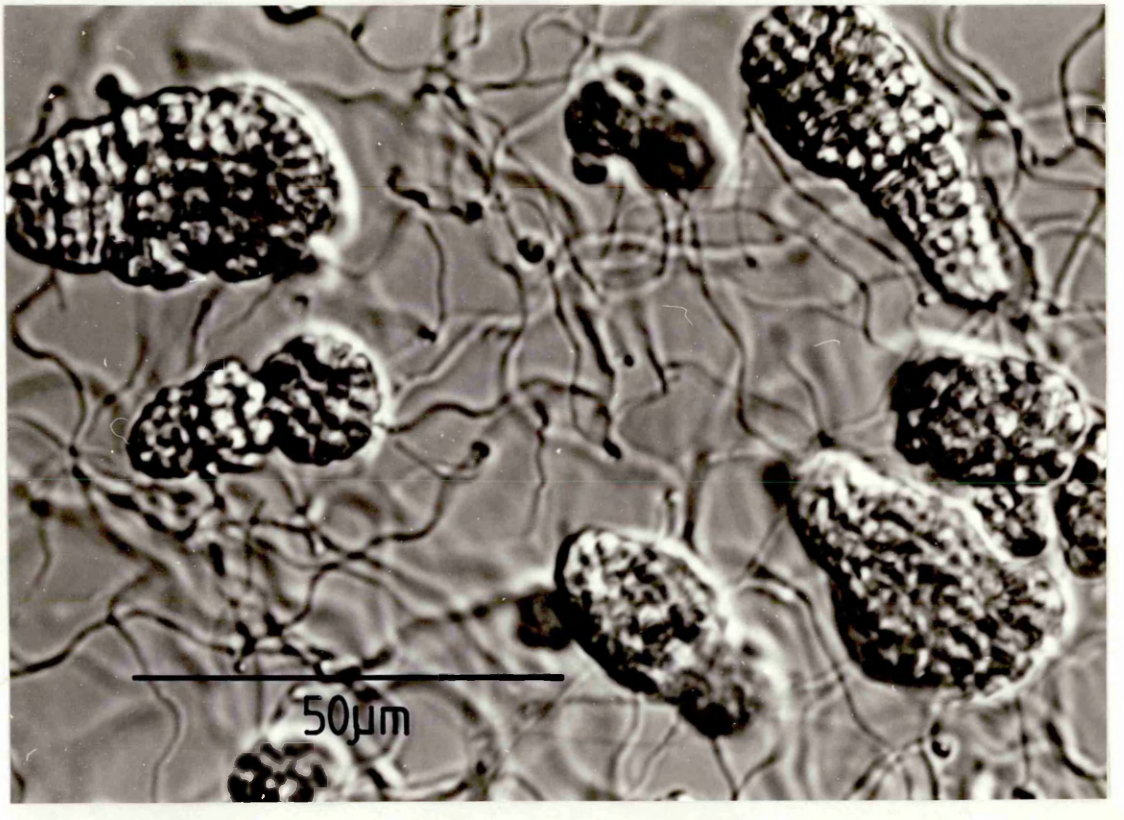

1mm

Plate 6: Sporangia from a colony of 1.2.5[Q](b).


50µm

to grow at all. Release of water-soluble pigments often became more pronounced (Table 11). Strains which were isolated on media other than BuCT but which grew slowly in liquid BuCT did not show improved growth on transfer to liquid media of the same composition (minus agar) as the isolation medium. In many cases such isolates were eventually lost, usually due to contamination. There were no obvious reasons for the failure of some isolates to grow in liquid culture and a number of isolates from both sp^+ and sp^- nodules behaved in this way. Reasons for the failure of isolates to grow when transferred to the liquid culture medium of the same composition as the solid medium on which they were isolated and in many cases grew well are not known.

For those isolates which were successfully cultivated, confirmation that the organism was indeed *Frankia* was obtained by the inoculation and subsequent nodulation of host plant seedlings. All isolates nodulated host plants effectively and those strains which nodulated *A.rubra* always effectively nodulated *A. glutinosa* and vice versa (Table 12). In most cases nodulation was 100% but in some was much lower (eg 1.2.23[Bu], 1.2.20[Bu]). The reasons for this were not clear but seedlings were used as test plants throughout and it is possible that genetic variation may have resulted in some seedlings being more sensitive to adverse conditions during infection than others e.g. elevated temperatures in the greenhouse.

Table 11: Pigmentation and growth of isolates on isolation and in liquid culture (BuCT).

Isolate	Pigmentation on isolation	Pigmentation In BuCT	Relative growth rate in BuCT[1]
1.1.1[BuC]	white	white	++
1.1.2[Q]	pink	pale yellow	++
1.1.4[Bu]	white	white	++
1.1.4[F]	white	white	++
1.1.5[F]	white	white	++
1.1.5[Bu]	white	white	++
1.1.7[Bu]	white	pale yellow	++
1.1.7[F]	white	pale yellow	++
1.1.11[P^-]	white	white	0
1.1.8[Bu]	white	white	+++
1.1.10[Bu]	white	white	0
1.1.14[Bu]	white	white	+++
1.1.14[Q]	white	white	++
1.2.3[Bu]	white	white	+
1.2.4[Bu]	white	yellow	+
1.2.5[P^+]	pale pink	pale yellow	++
1.2.5[Q] (a)	pale pink	white	0
1.2.5[Q] (b)	pink	orange	+++
1.2.10[Bu]	white	white	0
1.2.22[Bu]	white	white	+
1.2.13[Bu]	pale pink	pale orange	+++
1.2.13[Q]	pale pink	pale orange	+++
1.2.15[Bu]	white	white	+
1.2.15[Q]	white	white	0
1.2.23[Bu]	white	white	+
1.2.23[Q] (a)	white	white	+
1.2.23[Q] (b)	pink	orange	+++
1.2.18[Bu] (a)	white	white	0
1.2.18[Bu] (b)	white	white	+
1.2.19[Bu]	white	pale yellow	++
1.2.19[Q]	white	white	++
1.2.20[Bu]	white	white	++
1.2.20[Q]	white	white	+
1.4.2[Bu]	white	white	+++
1.4.2[BuCT]	white	white	+++
1.4.2[Q]	pink	pale pink	+++
1.4.2[P^+]	white	pale pink	+++
2.1.1[Bu]	white	white	0
2.1.2[F]	white	white	++

[1] Scale + to +++; 0 = no growth.

Table 12: Infectivity of *Frankia* isolates.

Isolate	Spore occurrence in nodules from which isolate obtained	Inoculated species[1]	
		A. glutinosa	*A. rubra*
1.1.1[BuC]	ND	12/12	8/8
1.1.2[Q]	-	10/10	9/9
1.1.4[Bu]	-	7/7	9/9
1.1.4[F]	-	9/10	10/10
1.1.5[F]	+/-	10/10	8/8
1.1.5[Bu]	+/-	9/9	7/8
1.1.7[Bu]	-	10/10	9/9
1.1.7[F]	-	9/9	12/12
1.1.11[P^-]	-	α	α
1.1.8[Bu]	-	13/14	8/8
1.1.10[Bu]	-	α	α
1.1.14[Bu]	-[2]	9/9	3/3
1.1.14[Q]	-[2]	10/10	9/9
1.2.3[Bu]	-	13/13	8/8
1.2.4[Bu]	-	7/7	10/10
1.2.5[P^+]	+	13/13	7/7
1.2.5[Q] (a)	+	α	α
1.2.5[Q] (b)	+	12/12	8/8
1.2.10[Bu]	-	α	α
1.2.22[Bu]	+/-	6/6	2/8
1.2.13[Bu]	-[2]	8/8	11/11
1.2.13[Q]	-[2]	7/7	12/12
1.2.15[Bu]	-[2]	6/6	10/10
1.2.15[Q]	-[2]	α	α
1.2.23[Bu]	-	4/8	2/8
1.2.23[Q] (a)	-	α	α
1.2.23[Q] (b)	-	11/11	8/8
1.2.18[Bu] (a)	+	α	α
1.2.18[Bu] (b)	+	3/10β	6/10β

Table 12 continued:

Isolate	Spore occurrence in nodules from which isolate obtained	Inoculated species[1]	
		A. glutinosa	*A. rubra*
1.2.19[Bu]	-	4/4	3/3
1.2.19[Q]	-	12/12	9/9
1.2.20[Bu]	-	2/4	2/10
1.2.20[Q]	-	4/4	5/5
		A. incana	
1.4.2[Bu]	+	ϵ	
1.4.2[BuCT]	+	ϵ	
1.4.2[Q]	+	ϵ	
1.4.2[P$^+$]	+	ϵ	
		H. rhamnoides	
2.1.1[Bu]	-	α	
2.1.2[F]	-	7/7	

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[1] Figures shown are for the number of plants effectively nodulated/number of surviving plants 15 weeks after inoculation. All nodules were spore negative. Control plants showed no nodulation.
[2] Not possible to observe spore cells. However, nodules were extremely small and poorly developed.
α Isolate could not be sub-cultured.
β Isolate contaminated subsequent to confirmation of ability to reinfect plants.
ϵ Infectivity confirmed by K. Huss-Danell, University of Umeå, Sweden.

3.2.0 Characterisation of Strains

3.2.1 *In vivo* spore production

Frankia was isolated from a number of sites. However, although strains were isolated from sites containing sp^+ nodules all the nodules which developed as a result of inoculation with the isolated strains were sp^-. The reasons for this are unclear.

3.2.2 Utilisation by strains of carbon sources for growth

Strains differed in their ability to utilise, for growth, a number of different carbon sources (Tables 13 to 15). Tween, acetate and propionate were each utilised by a number of strains and in some cases the presence of Tween was necessary before acetate or propionate could be utilised. A number of strains, however, did not utilise all 3. Glucose and succinate were also utilised but by fewer strains and again in a number of cases only in the presence of Tween. In general, growth when these carbon sources were utilised was less than when other carbon sources were utilised. Of the strains that did utilise glucose and succinate, all utilised one or more of the other carbon sources also.

Growth in a nitrogen-free medium, with propionate as a carbon source, was not ubiquitous . With the exception of 1.1.5[Bu], however, only those strains that utilised propionate in the presence

Table 13: Utilisation of carbon sources for growth by a number of *Frankia* strains.[1]

	Carbon Source.											
	Tween	Glucose		Acetate		Succinate		Propionate		Bu-N	
		-	+	-	+	-	+	-	+	-	+[2]
Strain											
1.1.1[BuC]	0	0	0	0	1	0	1	1	α	1	α
1.1.2[Q]	2	2	α	1	α	2	α	2	α	1	α
1.1.4[Bu]	2	0	2	0	2	0	2	2	α	2	α
1.1.4[F]	0	0	0	0	0	0	0	0	0	0	0
1.1.5[F]	0	0	0	0	0	0	0	0	0	0	0
1.1.5[Bu]	0	0	0	0	0	0	0	0	0	1	α
1.1.7[Bu]	0	0	0	0	0	0	0	0	0	0	0
1.1.7[F]	0	0	0	0	0	0	0	1	α	1	α
1.1.8[Bu]	1	1	α	0	1	1	α	0	1	0	1
1.1.14[Bu]	2	0	2	2	α	1	α	1	α	1	α
1.1.14[Q]	ND	0	3	3	α	0	3	0	3	0	3
1.2.3[Bu]	0	0	0	0	0	0	0	0	0	0	0
1.2.4[Bu]	0	0	0	0	0	0	0	0	0	0	0
1.2.5[P+]	3	0	3	3	α	0	3	3	α	3	α
1.2.5[Q](b)	3	1	α	4	α	2	α	4	α	3	α
1.2.13[Bu]	3	0	3	3	α	0	3	4	α	3	α
1.2.13[Q]	3	0	3	3	α	0	3	4	α	3	α
1.2.15[Bu]	0	0	0	0	0	0	0	0	0	0	0
1.2.23[Q](b)	3	0	3	3	α	0	3	3	α	3	α
1.2.18[Bu](b)	2	2	α	0	0	2	α	0	0	0	0
1.2.19[Bu]	2	1	α	0	0	2	α	2	α	1	α
1.2.19[θ]	2	1	α	1	α	1	α	1	α	2	α
1.2.20[Bu]	1	0	0	2	α	0	0	0	0	0	0
1.2.20[Q]	3	0	3	2	α	0	2	0	0	0	2
ArI3[3]	3	0	3	3	3	3	3	3	3	2	3
ArI4	2	2	1	0	1	0	0	2	2	2	1
ArI5[3]	3	0	0	0	2	0	0	0	2	0	2
Agn1C12	0	0	0	0	2	0	0	0	0	0	2

α Not determined.

[1] See 2.2.2 (p.48) for details of scoring.

[2] + indicates with Tween added, - indicates without Tween added.

[3] In these cases growth was clearly visible in control tubes which contained no carbon source.

Table 14: Carbon sources utilised by a number of *Frankia* strains.

Carbon source	Strains
Tween	1.1.2[Q], 1.1.4[Bu], 1.1.8[Bu], 1.1.14[Bu] 1.2.5[P+], 1.2.5[Q](b), 1.2.13[Bu], 1.2.13[Q], 1.2.23[Q](b), 1.2.18[Bu](b), 1.2.19[Bu], 1.2.19[Q], 1.2.20[Bu], 1.2.20[Q], ArI3, ArI4, ArI5.
Glucose	1.1.2[Q], 1.1.8[Bu], 1.2.5[Q](b), 1.2.18[Bu](b), 1.2.19[Bu], ArI4,.
Acetate	1.1.2[Q], 1.1.14[Bu], 1.1.14[Q], 1.2.5[P+], 1.2.5[Q](b), 1.2.13[Bu], 1.2.13[Q], 1.2.23[Q](b), 1.2.19[Q], 1.2.20[Bu], ArI3.
Succinate	1.1.2[Q], 1.1.8[Bu], 1.1.4[Bu], 1.2.5[Q](b), 1.2.18[Bu](b), 1.2.19[Bu], 1.2.19[Q], ArI3.
Propionate	1.1.1[BuC], 1.1.2[Q], 1.1.4[Bu], 1.1.7[F], 1.1.14[Bu], 1.2.5[P+], 1.2.5[Q](b), 1.2.13[Bu], 1.2.13[Q], 1.2.23[Q](b), 1.2.19[Bu], 1.2.19[Q], ArI3, ArI4.
None of those tested	1.1.4[F], 1.1.5[F], 1.1.5[Bu], 1.1.7[Bu], 1.1.7[F], 1.2.3[Bu], 1.2.4[Bu], 1.2.15[Bu], Agn1c12.

Table 15: Range of carbon sources utilised by a number of *Frankia* strains.

Group	Carbon Source(s) utilised	Strain(s)
1	Tween alone	1.2.20[Q], ArI5
2	Acetate alone	1.1.14[Q]
3	Propionate alone	1.1.1[BuC], 1.1.7[F]
4	Tween and acetate	1.2.20[Bu]
5	Tween and propionate	1.1.4[Bu]
6	Tween, glucose and succinate	1.1.8[Bu], 1.2.18[Bu](b)
7	Tween, glucose and propionate	ArI4
8	Tween, acetate and propionate	1.2.5[P+], 1.2.13[Bu], 1.2.13[Q]
9	Tween, glucose, acetate, succinate, and propionate	1.1.2[Q], 1.2.5[Q](b)
10	Tween, glucose, propionate and succinate	1.2.19[Bu]
11	Tween, acetate, succinate and propionate	1.1.14[Bu], 1.2.19[Q], ArI3
12	None of those tested	1.1.4[F], 1.1.5[F], 1.1.5[Bu], 1.1.7[Bu], 1.1.7[F], 1.2.3[Bu], 1.2.4[Bu], 1.2.15[Bu], Agn1c12.

of combined nitrogen would do so in its absence. Where propionate was not utilised but Tween was and Tween was included in Bu-N medium growth occurred in all cases with the exception of 1.2.18[Bu](b) and 1.2.20[Bu].

It is possible to categorise strains into 12 distinct groups dependant upon the range of carbon sources they were able to utilise. There are no obvious connections between the range of carbon sources utilised and site or isolation. In general, however, strains isolated from *A. glutinosa* utilised fewer carbon sources and grew less well than strains isolated from *A. rubra*.

A particularly interesting result is the ability of strains ArI3 and ArI5 to grow in control medium to which no carbon source had been supplied; it was possible to detect this by comparison with tubes containing carbon sources in which little, if any, growth had occurred. This does, however, effectively remove any controls for comparison and so the results for the utilisation of any carbon source by these strains must be treated with caution. Clearly, however, as there are a number of treatments in which no visible growth occurred this carbon fixation may be inhibited by the presence of some compounds.

Interestingly, a number of strains failed to utilise any of the carbon sources tested and of these 9 strains 6 were isolated from *A. glutinosa*. All these strains were, however, able to grow satisfactorily in BuCT. As all carbon sources in this medium were tested other than casamino acids it is probable that they either used

these or that there was some synergism between the different compounds present. Detection of utilisation was, however, limited to visual comparisons with controls and it is possible that a low growth may have been overlooked. The possibility of a non-viable culture was discounted since the addition of BuCT to cultures which had previously shown no growth in all cases stimulated growth. Another possibility is that some small growth may have occurred in control tubes due to fixation of CO_2 so that a low rate of growth in the experimental tubes may have been overlooked.

3.2.2 Effectivity of strains for nodulation and nitrogen fixation in *Alnus rubra*

Variation in effectivity between Frankiae was shown by differences in the growth of *A. rubra* when inoculated with either different sources of crushed nodules or with different isolated *Frankia* strains and grown under similar conditions in either perlite or water culture in the absence of combined nitrogen.

In the assessment of strain effectivity analyses of statistical significance between different host plant/strain combinations were made using plant dry weight only since time did not permit the assay of large numbers of samples for total nitrogen. In general, however, plant nitrogen content correlated well with plant dry weight, even when plants were grown under different conditions (Figure 7). In addition, when individual measurements of plant nitrogen were made differences between plants were found to be small as indicated by the

low standard errors in Table 46. Differences in the nitrogen content of plant material did occur, however, as is illustrated by the relative differences between plant dry weight and plant nitrogen in plants inoculated with strains ArI4 and 1.1.1[BuC] in batch 1 (Figure 7 , Table 21). Consequently analyses of total nitrogen were made on samples of bulked plant material from individual strain assessments in each experiment .

Tables 16 and 17 show the relative effects on the growth of *A. rubra* of inoculation with 3 different sources of *A. rubra* crushed nodules. There were significant effects of inoculum source on plant dry weight ($p < 0.010$), nodule dry weight ($p < 0.050$), nodule number ($p < 0.001$), ratios of root to shoot dry weight ($p < 0.050$) and of plant / nodule dry weight ($p < 0.001$) which closely approximates to nodule specific activity in N fixation (ie. plant nitrogen / nodule dry weight). Plants inoculated with crushed nodules from McNab's Farm had a considerably greater dry weight than plants inoculated with either of the other sources and the ratio of root to shoot dry weight was also reduced. Plants inoculated with crushed nodules from Timberlands, the other sp^- strain, however, had a greatly reduced nodule dry weight and ratios of nodule / plant dry weight were almost identical in plants inoculated with either source of sp^- crushed nodules. Plants inoculated with sp^+ crushed nodules from Corvallis, however, although not significantly different in dry weight to plants inoculated with crushed nodules from Timberlands had a higher nodule dry weight and the plant / nodule dry weight ratio for these plants was only approximately one third that of plants inoculated with crushed nodules from either of the sp^- sources, indicating reduced

Figure 7: Relationship between plant dry weight and plant nitrogen in inoculated *A. rubra* grown under different conditions.

Plants were grown for (□) 225 days in Perlite in the greenhouse after inoculation with crushed nodules, (○) 65 days in Perlite in controlled environment cabinets after inoculation with *Frankia* strains a, ArI4; c, ArI5; e, ArI3; t, CpI1; u, AvCI1 (●) 96 days in Perlite in controlled environment cabinets after inoculation with a, ArI4; b, 1.2.5[Q](b); c, ArI5; d, 1.2.5[P$^+$]; e, ArI3; f, crushed nodules; g, 1.1.1[BuC]; h, 1.1.8[Bu]; i, Agn1c12; j, 1.1.2[Q]; k, 1.1.4[F]; l, 1.1.5[F]; m, 1.2.23[Q]; n, 1.1.14[Q]; o, 1.2.20[Bu]; p, 1.2.13[Q]; q, 1.2.13[Bu]; r, 1.2.19[Q]; s, 1.2.15[Bu].

Figures after the letter 'a' indicate a batch number.

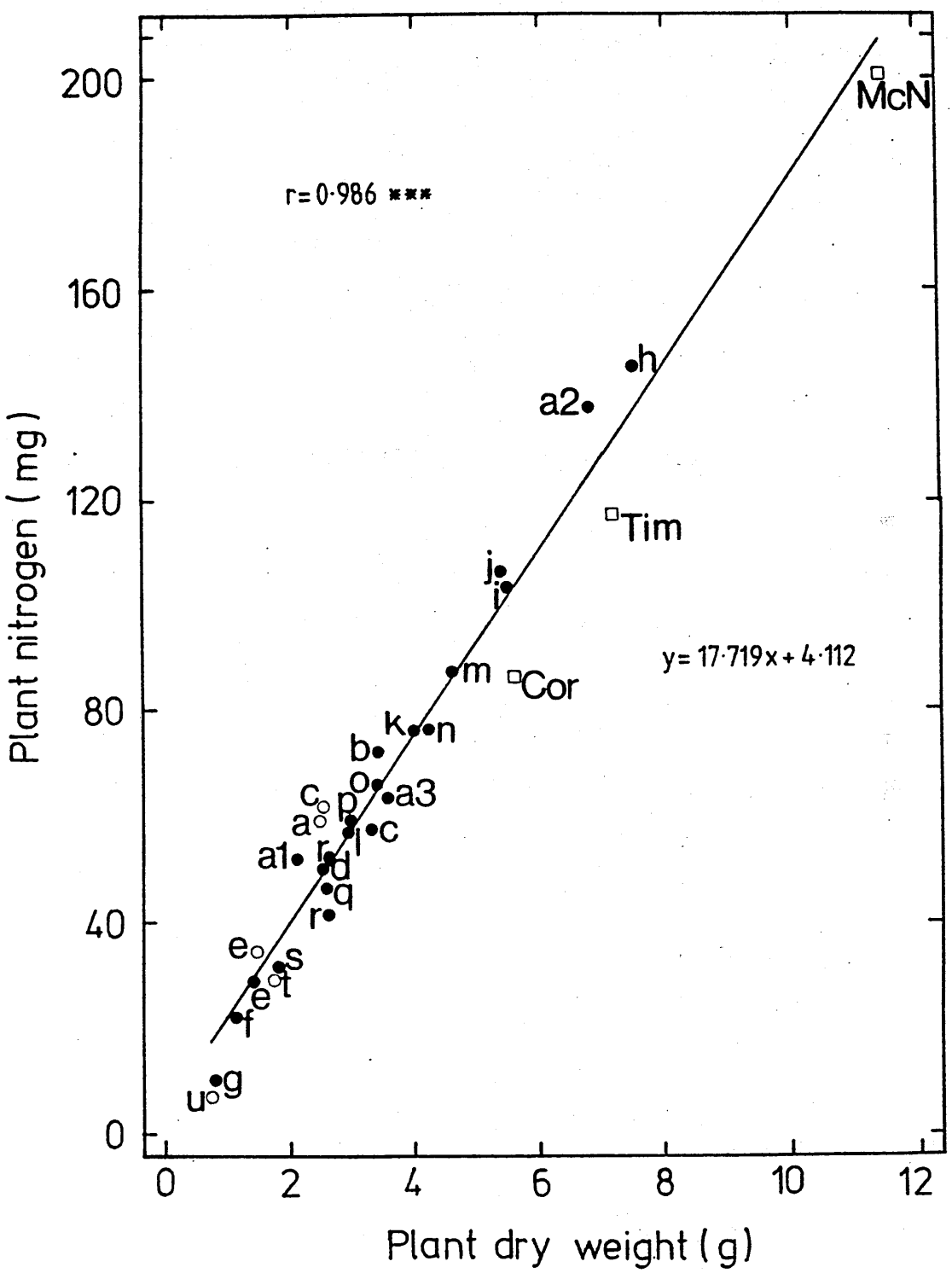

r=0·986 ***
y= 17·719x + 4·112
McN
Tim
Cor
h
a2
j
i
m
k
n
b
o
a3
c
p
a
l
a1
r
d
q
e
s
t
f
u
g
Plant nitrogen (mg)
Plant dry weight (g)
0
40
80
120
160
200
0
2
4
6
8
10
12

Table 16: Growth of *A. rubra* inoculated with crushed nodule preparations from different provenances of *A. rubra*.

	Source of inoculum			
	Corvallis (sp^+)	McNabs(sp^-)	Timberlands(sp^-)	SE
Plant d.w. (g)	5.6 a	11.4 b	7.1 a	1.12
Nodule d.w.(g)	0.195 a	0.145 ab	0.091 b	0.0226
Plant nodule no.	145 a	12 b	7 b	12.8
Plant / nodule d.w.	28.4 a	82.6 b	81.4 b	4.54
Ratio root to shoot d.w.	0.67 ab	0.57 b	0.77 a	0.049

Figures are means of 8 replicates. Means followed by the same letter are not significantly different (p=0.05) from one another using Duncan's Multiple Range Test. Where means are not followed by a letter Analysis of Variance showed no significant effect (p=0.05) of strain on the parameter. Spore production or absence confirmed by sectioning. Nodules were of same spore type as inoculum. SE indicates standard error of treatment mean. Analysis of Variance table is shown in Table 16S, Appendix 4.

Table 17: Nitrogen content of *A. rubra* inoculated with crushed nodule inocula.

Inoculum Source	N(mg)g plant $d.w.^{-1}$ [1]	Plant N (mg)	Plant N / nodule d.w.
McNabs	17.64	201.10	1.39
Timberlands	16.51	117.22	1.29
Corvallis	15.46	86.58	0.44

[1] Figures are means of duplicate determinations.

nodule specific activity. The figures for nitrogen content in Table 17 confirm these relationships between sources when comparisons are of plant total nitrogen. It would appear, therefore, on the basis of these results that the sp^+ nodules, produced by inoculation with crushed nodule inoculum from a sp^+ nodule source, were considerably less effective in nitrogen fixation than sp^- nodules produced by inoculation with sp^- nodule sources.

Differences in growth also resulted when *A. rubra* was inoculated with isolated *Frankia* strains and grown in water culture. Table 18 shows significant differences in dry weight ($p < 0.001$) and differences in nitrogen content of plants inoculated with different strains. This experiment was performed to provide comparative information on the symbiosis of these srains with *A. rubra* to permit selection of a suitable strain for inclusion as a comparator combination in a series of more detailed strain analysis experiments and for use in the competition experiment, the results of which are described in 3.3.4. In both cases it was necessary to identify a strain which would give good nodulation and support adequate growth of seedlings. It is clear from the results that ArI4 and ArI5 showed these characteristics and they were utilised in later experiments as the comparator strain for analysis of different strain : host plant combinations and for strain competition studies respectively.

The more detailed experiments now described were designed to evaluate the capacity of strains for symbiotic nitrogen fixation in *A. rubra*. The large number of strains to be tested made it impractical for them all to be compared at the one time and for this reason they were

Table 18: Growth of *A. rubra* inoculated with different *Frankia* strains and grown in water culture.

Strain	Plant d.w.(g)	N(mg)g plant d.w^{-1}. ± S.E
ArI5	2.52 a	23.7 ± 1.15
ArI4	2.47 a	23.8 ± 0.67
CpI1	1.71 ab	17.1 ± 0.79
ArI3	1.44 bc	23.6 ± 2.11
AvcI1	0.74 c	9.3 ± 0.60

Plant dry weights are the means of 11 to 15 plants. Means followed by the same letter are not significantly different from one another using Duncan's Multiple Range Test. Where means are not followed by a letter Analysis of Variance showed no significant effect (p=0.05) of strain on the parameter. Nitrogen figures are means of duplicate determinations from each of 5 replicate pots. Analysis of Variance table is shown in Table 18S, Appendix 4.

evaluated in three separate batches. Comparison of each host plant/strain combination with the comparator combination with strain ArI4, which was included in each batch, made inter-batch comparisons possible by compensating for any differences in conditions from batch to batch.

The results are shown in Tables 19, 20 and 21. The considerable differences observed in nodulation time, plant dry weight and plant nitrogen, nodule dry weight, nodule number, plant / nodule dry weight and plant nitrogen / nodule dry weight of ArI4 inoculated plants in different batches clearly illustrate the importance of inclusion of a comparator in these experiments. Some of the differences were surprisingly large; for example, plant dry weight in batch 2 was approximately 3 times as great as in batch 1. There were no apparent reasons for such large differences and it is difficult to speculate. It seems unlikely, however, that genetic differences due to the use of seedling rather than clonal plants would have given rise to such large differences since all *A. rubra* fruits from which plants were grown was collected from the same provenance. Despite this differences between similarly inoculated plants were small enough for significant differences to be detected between the growth of plants inoculated with different strains in the same batch; none of the control plants became nodulated.

The distribution of nodules on the root system varied considerably between plants inoculated with different strains. Interestingly, the only plants in which nodulation was restricted to the root crown were all inoculated with strains isolated from nodules growing outside

Table 19: Nodulation of *A. rubra* inoculated with *Frankia* strains (Batches 1 to 3).

Batch 1

Strain	Nodulation time (days)	Percentage nodulation	Nodule distribution[1]
ArI4	28	100	1
1.2.5[Q] (b)	28	100	2
ArI5	35	100	2
1.2.5[P+]	28	100	2
ArI3	28	100	1
Crushed nodules	35	83	1
1.1.1[BuC]	28	100	3

Batch 2

Strain	Nodulation time (days)	Percentage nodulation	Nodule distribution[1]
ArI4	21	100	1
1.1.8[Bu]	21	100	2
Agn1C12	21	100	1
1.1.2[Q]	21	100	2
1.1.4[F]	21	100	2
1.1.5[F] (50%)	21	100	2
1.1.5[F] (100%)	21	100	2

Table 19 continued

Batch 3

Strain	Nodulation time (days)	Percentage nodulation	Nodule distribution[1]
ArI4	14	100	1
1.2.23[Q](b)	21	100	2
1.1.14[Q]	14	100	2
1.2.20[Bu]	14	100	2
1.2.13[Q]	21	100	3
1.2.13[Bu]	21	100	2
1.2.19[Q]	21	100	2
1.2.15[Bu]	21	88	2

[1] Nodule distribution is coded as follows: 1, confined to root crown; 2, confined to upper 30% of root system and 3, extends throughout upper 60 to 70% of root system.

Table 20: Growth of *A. rubra* inoculated with *Frankia* strains (Batches 1 to 3).

Batch 1

Strain	Plant d.w.(g)	Nodule d.w.(g)	Nodule number	Ratio root to shoot d.w.	Plant / nodule d.w.
ArI4	2.1 abc	0.043 ab	7 bc	0.28	50.0 d
1.2.5[Q](b)	3.4 d	0.095 d	14 ab	0.31	36.3 b
ArI5	3.3 cd	0.083 cd	17 a	0.29	39.0 bc
1.2.5[P+]	2.5 bcd	0.065 abc	18 a	0.30	39.0 bc
ArI3	1.4 ab	0.030 a	8 bc	0.24	46.4 cd
Crushed nodules	1.1 a	0.031 a	3 c	0.36	27.1 a
1.1.1[BuC]	0.8 a	0.041 ab	42 d	0.35	20.0 a
S.E.	0.42	0.0099	2.3	0.028	3.23

Batch 2

Strain	Plant d.w.(g)	Nodule d.w.(g)	Nodule number	Ratio root to shoot d.w.	Plant / nodule d.w.
ArI4	6.8 c	0.112	12 d	0.24	63.7 c
1.1.8[Bu]	7.5 c	0.131	64 e	0.24	65.8 c
Agn1C12	5.5 bc	0.107	16 cd	0.25	53.9 b
1.1.2[Q]	5.4 bc	0.118	109 f	0.22	48.7 b
1.1.4[F]	4.0 ab	0.134	44 a	0.23	28.8 a
1.1.5[F] (50%)'	3.2 ab	0.110	30 abc	0.22	24.8 a
1.1.5[F] (100%)	2.9 ab	0.124	33 ab	0.22	23.9 a
1.1.5[F] (17%)	2.6 a	0.114	21 bcd	0.28	20.8 a
S.E.	0.85	0.021	4.8	0.04	3.23

Table 20 continued

Batch 3

Strain	Plant d.w. (g)	Nodule d.w. (g)	Nodule number	Ratio root to shoot d.w.	Plant / nodule d.w.
ArI4	3.6 cdef	0.055 a	12 b	0.23 ab	73.7 e
1.2.23[Q](b)	4.6 f	0.088 b	19 a	0.24 a	51.1 abcd
1.1.14[Q]	4.2 ef	0.060 a	23 a	0.27 d	72.0 e
1.2.20[Bu]	3.4 bcde	0.053 a	9 b	0.23 ab	68.9 e
1.2.13[Q]	2.9 bcd	0.055 a	33 c	0.16 c	53.4 bcd
1.2.13[Bu]	2.6 abc	0.054 a	22 a	0.20 abc	49.3 ab
1.2.19[Q]	2.6 ab	0.053 a	10 b	0.18 bc	50.0 abc
1.2.15[Bu]	1.8 a	0.047 a	11 b	0.21 abc	38.0 a
S.E.	0.39	0.0074	2.1	0.017	4.30

Figures are means of 12 replicates, except in the case of crushed nodules where means are of 10 replicates. Means in the same column followed by the same letter are not significantly different ($p < 0,050$) from one another using Duncan's Multiple Range Test. Where means are not followed by a letter Analysis of Variance showed no significant effect ($p < 0,050$) of strain on the parameter. S.E. indicates standard error of the treatment mean. Mean plant dry weight values for non-inoculated control plants ranged from 0,004 to 0,008g, 0,025 to 0,071g and 0,010 to 0,040g in batches 1, 2 and 3 respectively. Analysis of Variance tables are shown in Table 20S, Appendix 4.

Using mean figures from each set of inoculated plants a significant ($r = 0,975$, $p < 0,001$) correlation of plant dry weight to nodule dry weight and a significant ($r = 0,951$, $p < 0,010$) correlation of plant nitrogen to nodule dry weight was obtained in batch 1. Similarly, in batch 3 significant correlations were obtained between both plant dry weight ($r = 0,842$, $p < 0,050$), plant nitrogen ($r = 0,837$, $p < 0,050$) and nodule dry weight. In batch 2, however, no significant correlations were observed between either plant dry weight ($r = -0,041$) or plant nitrogen ($r = -0,020$) and nodule dry weight. In batches 2 and 3 there was no correlation between plant dry weight ($r = 0,332$ and $r = 0,0,077$ respectively) or plant nitrogen ($r = 0,381$ and $r = 0,158$ respectively) and nodule number. In batch 1, however, there was a significant correlation between plant dry weight and nodule number ($r = 0,826$, $p < 0,050$) but not with plant nitrogen ($r = 0,754$). Strains isolated from *A. glutinosa* were not included in the analysis of batches 1 and 3 and strains isolated from *A. rubra* not included in the analysis of batch 2.

[1] Figure in brackets indicates percentage of 5,25µg *Frankia* protein seedlings were inoculated with.

Table 21: Nitrogen content of *A. rubra* inoculated with *Frankia* strains (Batches 1 to 3).

Batch 1

Strain	N(mg)g plant d.w.$^{-1}$	Plant N(mg)^{2}	Plant N / nodule d.w.
ArI4	22.24	46.26	1.08
1.2.5[Q](b)	21.41	72.15	0.76
ArI5	17.68	57.81	0.70
1.2.5[P+]	20.34	50.44	0.78
ArI3	20.94	28.69	0.96
Crushed nodules	20.16	22.18	0.72
1.1.1[BuC]	12.85	10.28	0.25

Batch 2

Strain	N(mg)g plant d.w.$^{-1}$	Plant N (mg)^{2}	Plant N / nodule d.w.
ArI4	20.35	137.77	1.23
1.1.8[Bu]	19.43	144.75	1.11
Agn1C12	18.83	103.00	0.96
1.1.2[Q]	19.77	106.36	0.90
1.1.4[F]	19.31	76.27	0.57
1.1.5[F](50%)	16.94	53.36	0.49
1.1.5[F](100%)	19.71	56.76	0.46
1.1.5[F](17%)	17.54	44.73	0.39

Table 21 continued

Batch 3

Strain	N(mg)g plant d.w.$^{-1}$ [1]	Plant N (mg)[2]	Plant N / nodule d.w.
ArI4	18.69	63.35	1.15
1.2.23[Q](b)	18.89	86.93	0.99
1.1.14[Q]	17.96	76.15	1.27
1.2.20[Bu]	19.19	65.25	1.23
1.2.13[Q]	20.46	59.54	1.08
1.2.13[Bu]	18.00	46.26	0.86
1.2.19[Q]	20.12	51.51	0.97
1.2.15[Bu]	17.86	31.26	0.67

[1] Figures are means of duplicate determinations.

[2] Calculated by multiplying the mean plant dry weight for each strain by the mean nitrogen content per gram plant dry weight.

Britain. One hundred percent of all inoculated plants were nodulated at harvest with the exception of those inoculated with a crushed nodule preparation where the figure was only 83% and 1.2.15[Bu] where only 88% of plants were nodulated.

Differences in nodule number per plant provide a measure of the relative infectivity of strains. The data of Table 19 shows that there were significant effects of strain on nodule number in all 3 batches ($p < 0.001$). However, there were no correlations between either plant dry weight or plant nitrogen and nodule number in batches 2 and 3. Plant size does not, therefore, seem to be a primary cause of differences in nodule number, which should usually reflect variations in infectivity between strains, characteristic for the conditions of these experiments. In batch 1 although there was a significant ($p < 0.050$) correlation of nodule number with plant dry weight a similar correlation was not observed with plant nitrogen. Given the absence of evidence to the contrary, differences in nodule number were unlikely to be attributable purely to or a determinant of differences in plant size and more likely represent a variation in the ability of strains for infection, characteristic of the strain under the conditions of these experiments.

There were significant effects of strain on plant dry weight in all batches ($p < 0.001$). Plant nodule dry weight varied significantly with strain in batches 1 and 3 ($p < 0.001$ and 0.050 respectively), which compared strains isolated mainly from *A. rubra* nodules. There were also differences in nodule specific activity as indicated by the significantly ($p < 0.001$) different ratios for plant / nodule dry

weight and the similar trends for ratios of plant nitrogen / nodule dry weight. However, the range of ratios for different strains was smaller in batches 1 and 3 than in batch 2 which compared strains isolated mainly from *A. glutinosa*. Here differences in plant dry weight were due mainly to differences in nodule specific activity. The highest ratio of plant / nodule dry weight for *A. rubra* inoculated with strains isolated from *A. glutinosa* (the heterologous association) was almost 3 times that of the lowest, compared to a range difference of only 1.8 times when strains isolated from *A.rubra* were inoculated onto *A. rubra* (the homologous association). In both batches 1 and 3 when plants inoculated with strains isolated from *A. glutinosa* (i.e. the heterologous associations) were excluded there were significant correlations between plant dry weight and nodule dry weight ($p < 0.001$ and 0.050 respectively) and plant nitrogen and nodule dry weight ($p < 0.010$ and 0.050 respectively). In general, therefore, nodule specific activity was relatively constant in such associations, increased nitrogen fixation being acieved primarily by an increase in nodule weight per plant. In the heterologous associations in batch 2, however, there were no such correlations between the above parameters and differences in nitrogen fixed per plant could be ascribed mainly to differences in the specific activity of nitrogen fixation of the nodules.

Significant differences in root to shoot ratio were only observed ($p < 0.001$) in batch 3, but within the homologous associations such differences were not large (ratios of 0.16 to 0.24).

The possibility that small differences in the dilution of the inoculum might affect results was examined in batch 2 (Table 20), where dilution of 1.1.5[F] inoculum by a factor of 2 or 6 had no significant ($p < 0.050$) effect on any of the measured or calculated parameters of plant growth. Small differences in the amount of inoculum applied are, therefore, unlikely to have affected growth significantly.

In batch 1, sets of plants were inoculated with either 1.2.5[Q](b) or 1.2.5[P+], strains isolated from the same nodules. There were significant ($p < 0.050$) differences in the nodule dry weight of inoculated plants but in all other aspects they were similar. Plants inoculated with crushed nodules (sp^+) from the same site as these two strains were isolated, however, developed nodules of lower specific activity than either strain. Nodule weight per plant was also reduced and nodules which developed were predominantly sp^-. In batch 2 when plants were inoculated with either 1.1.2[Q], 1.1.4[F] or 1.1.5[F], strains all isolated from the same site on different occasions, growth of plants inoculated with the last two strains, which were isolated on FMC+P medium, was similar. However, plants inoculated with strain 1.1.2[Q], isolated on Qmod, developed 2 to 3 times the number of nodules of those inoculated with the other 2 strains. The nodules formed were also almost twice as effective in nitrogen fixation, as indicated by the significant ($p < 0.050$) differences in the ratio of plant / nodule dry weight and corresponding differences in the ratio of plant nitrogen / nodule dry weight. Finally, in batch 3 it is interesting to compare *A. rubra* inoculated with 1.1.14[Q] and 1.2.15[Bu] isolated from *A.glutinosa* and *A. rubra* respectively at Rumster 9. Plant dry weight, nodule number,

and ratios of root / shoot dry weight and plant / nodule dry weight were all significantly ($p < 0.050$) greater in plants inoculated with the strain originally isolated from *A. glutinosa*. The plant nitrogen and plant nitrogen / nodule dry weight figures followed similar trends. Inoculation with the strain isolated from *A. rubra*, also gave rise to reduced nodule productivity per plant and nodule specific activity, as well as nodulating only 88% of plants. The effectivity of this strain was thus inferior in its symbiosis with *A. rubra* in every respect compared with the strain isolated from *A. glutinosa*.

Expression of the data for plants in each batch as a ratio of the appropriate data for the comparator symbiosis with strain ArI4 permits examination of general relationships between host plant / *Frankia* strain in all batches. Figures 8 and 9 show plant dry weight and plant nitrogen plotted against nodule dry weight after standardisation against the symb iotic performance of ArI4 with *A. rubra* by dividing by the appropriate value for that symbiosis. It is clear that a number of strains isolated from *A. rubra* were superior in effectivity to any strain isolated from *A. glutinosa*. Different contributions of nodule specific activity or nodule growth to overall effectivity in nitrogen fixation in the associations with *A. rubra* are also apparent (Figures 10 and 11). In the homologous associations there were significant ($p < 0.001$) correlations between plant dry weight, plant nitrogen and nodule dry weight; the ratios of plant dry weight and of plant nitrogen to nodule dry weight were relatively constant for all associations. In the heterologous associations, however, no such relationships were observed - the ratios of plant dry weight or plant nitrogen to nodule dry weight were not constant. There were no

Figure 8: Relationship between plant and nodule dry weight of *A. rubra* inoculated with *Frankia* in batches 1 to 3 standardised relative to growth with ArI4.

(■) indicates strains isolated from *A. rubra* (○) indicates strains isolated from *A. glutinosa*. Strain letters as in the legend to Figure 7(p.115). Line drawn is for A. rubra strains.

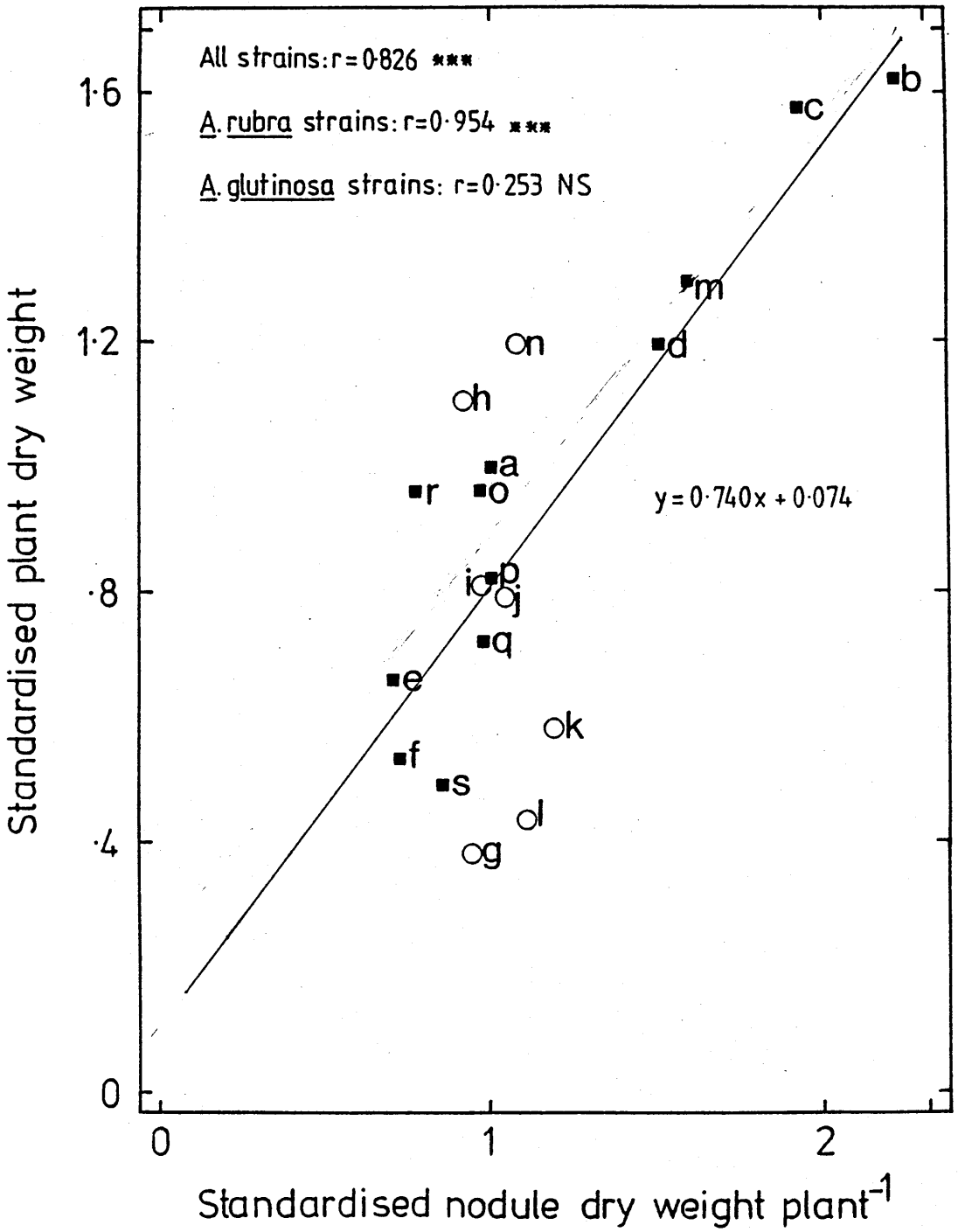

All strains: r = 0·826 ***
A. rubra strains: r = 0·954 ***
A. glutinosa strains: r = 0·253 NS
y = 0·740x + 0·074
Standardised plant dry weight
Standardised nodule dry weight plant⁻¹
0
·4
·8
1·2
1·6
1
2
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
s

Figure 9: Relationship between plant nitrogen and nodule dry weight of *A. rubra* inoculated with *Frankia* strains in batches 1 to 3 standardised relative to growth with ArI4.

(■) indicates strains isolated from *A. rubra* (○) indicates strains isolated from *A. glutinosa*. Strain letters as in the legend to Figure 7 (p.115). Line drawn is for A rubra strains.

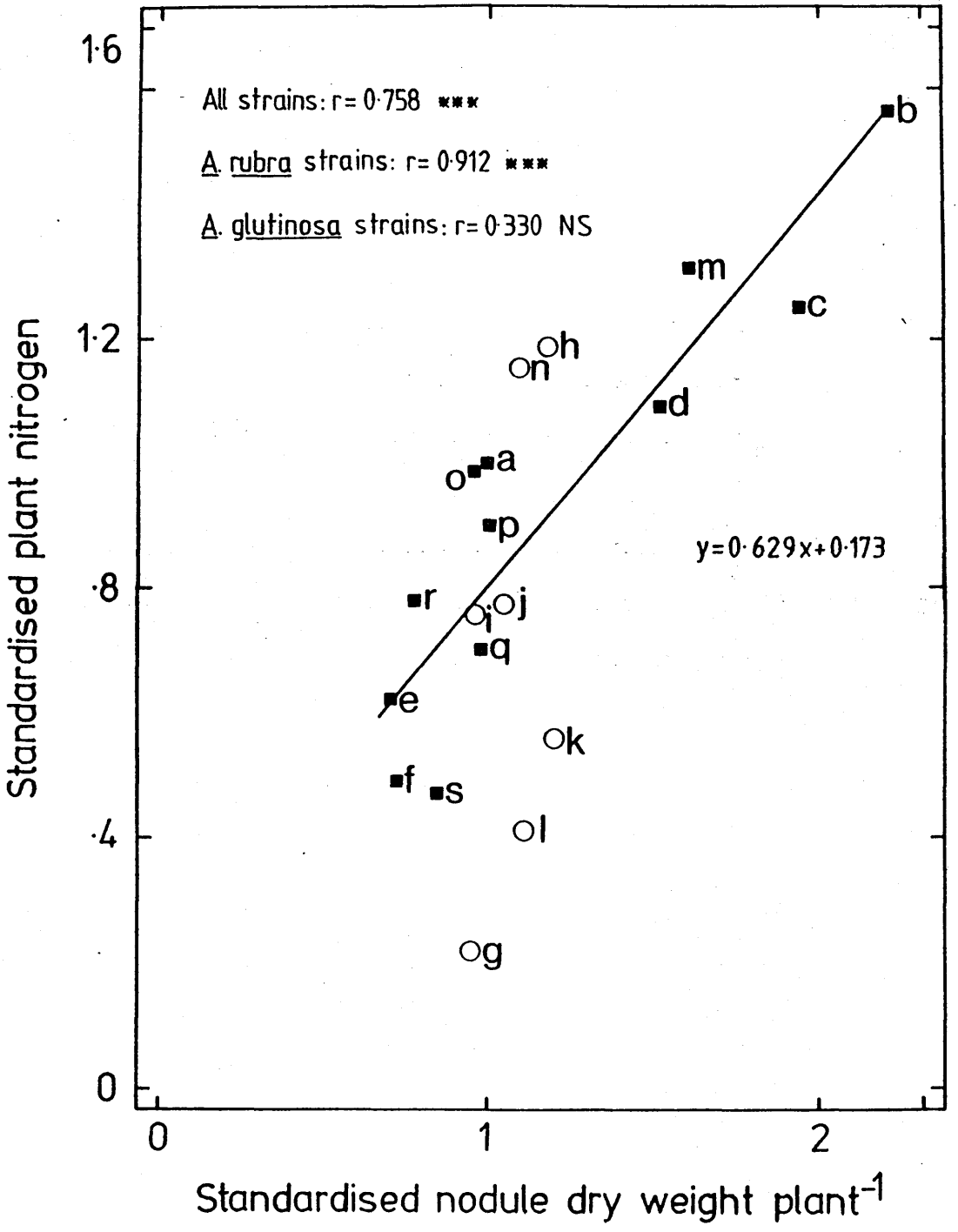

All strains: r = 0·758 ***
A. rubra strains: r = 0·912 ***
A. glutinosa strains: r = 0·330 NS
y = 0·629x + 0·173
Standardised plant nitrogen
Standardised nodule dry weight plant⁻¹
1·6
1·2
·8
·4
0
0
1
2
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
s

Figure 10: Relationship between plant nodule dry weight and nodule specific activity (plant nitrogen/nodule dry weight) standardised relative to ArI4 plants.

(■) indicates strains isolated from *A. rubra* (○) indicates strains isolated from *A. glutinosa*. Strain letters as in the legend to Figure 7(p.115).

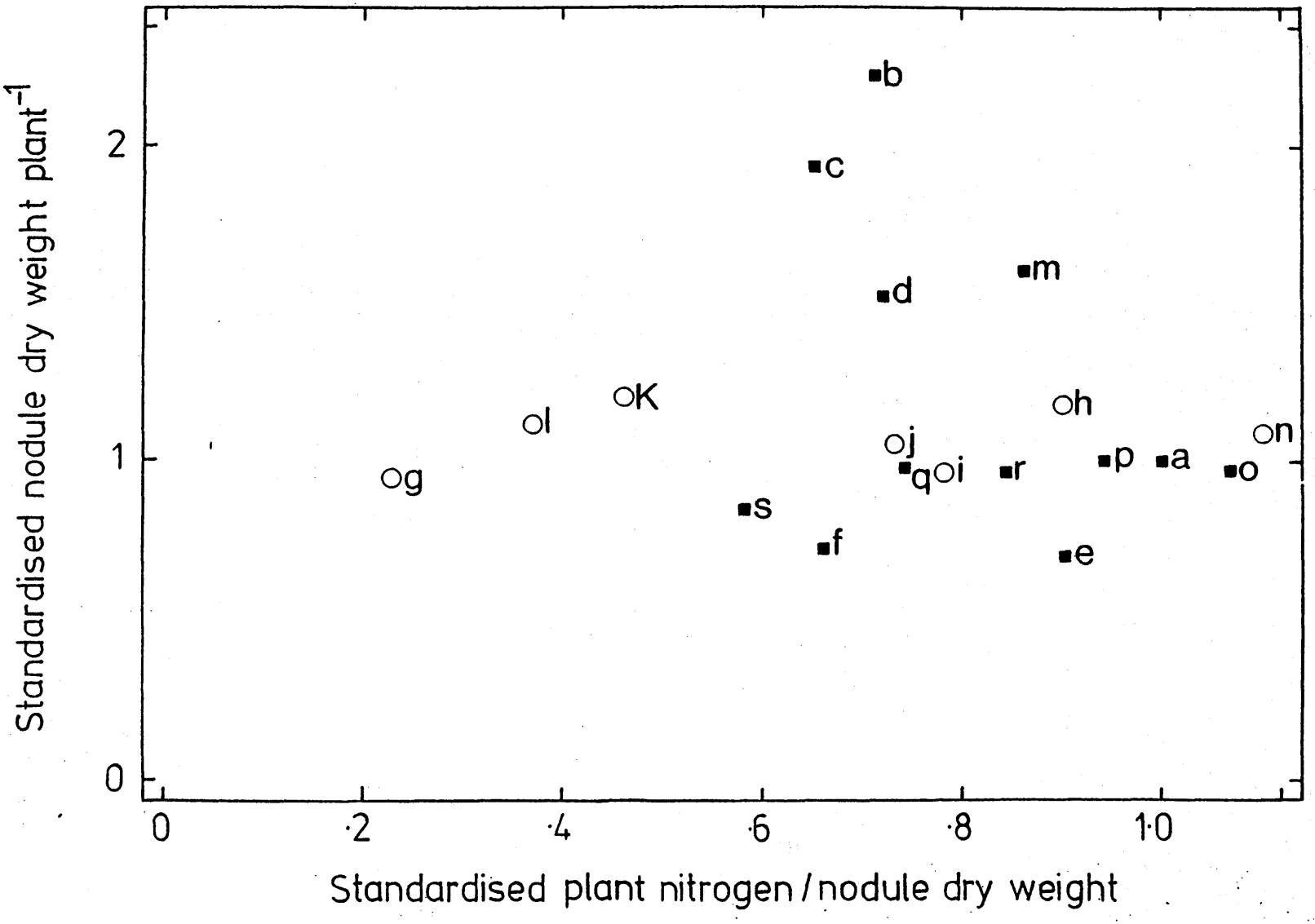
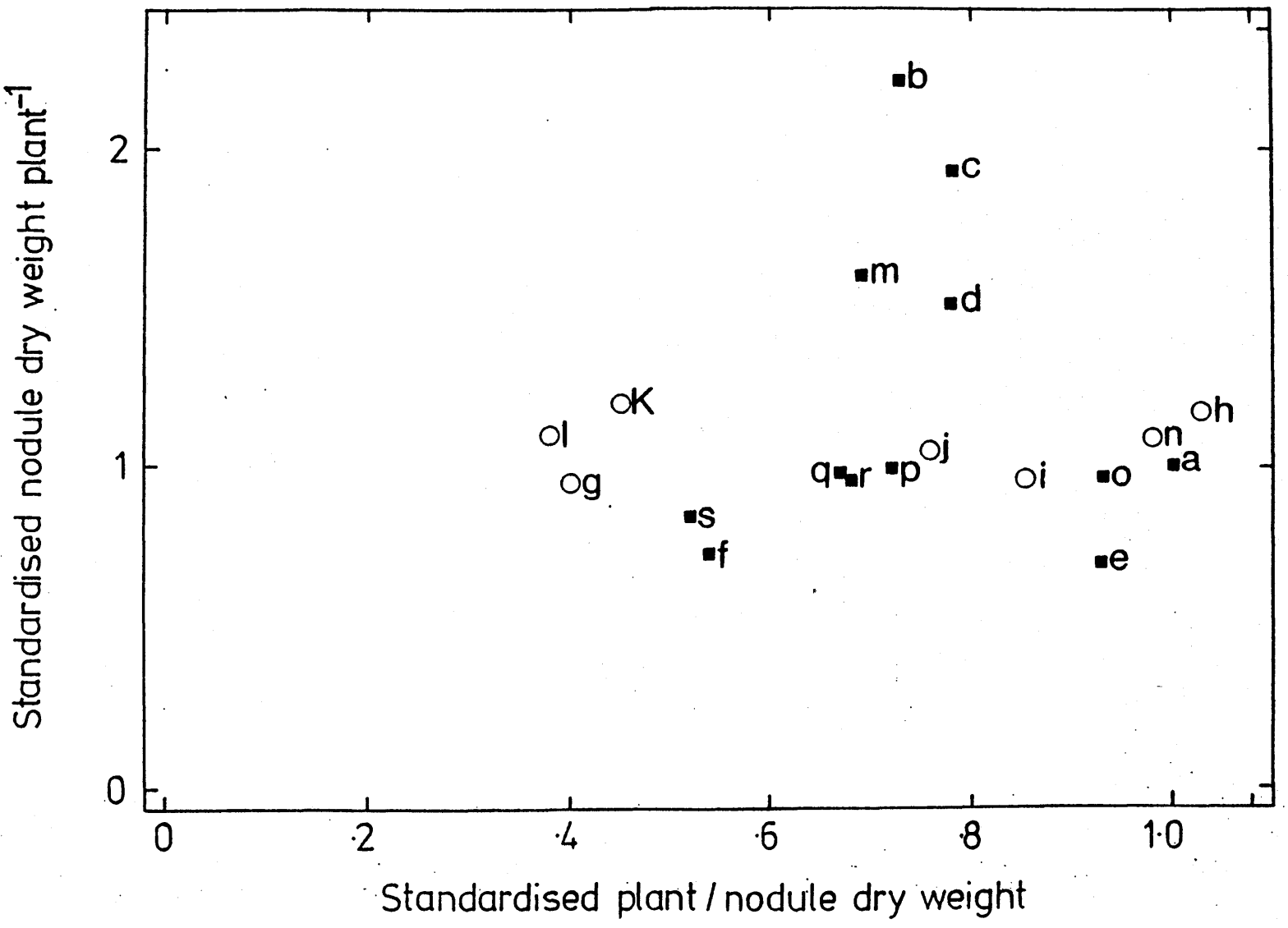

Standardised nodule dry weight plant^{-1}
2
1
0
·2
·4
·6
·8
1·0
Standardised plant nitrogen / nodule dry weight
b
c
d
m
K
l
h
j
n
g
q
i
r
p
a
o
s
f
e

Figure 11: Relationship between plant nodule dry weight and nodule specific activity (plant/nodule dry weight) standardised relative to ArI4.

(■) indicates strains isolated from *A. rubra* (○) indicates strains isolated from *A. glutinosa*. Strain letters as in the legend to Figure 7(p.115).


Standardised nodule dry weight plant^{-1}
2
1
0
b
c
m
d
K
l
g
h
n
a
j
q
r
p
i
o
s
f
e
0
·2
·4
·6
·8
1·0
Standardised plant / nodule dry weight

correlations for either the homologous or heterologous associations between plant dry weight or plant nitrogen and nodule number.

In batch 1, nodulation of seedlings inoculated with *A. rubra* crushed nodules was lower (83%) compared with the 100% nodulation achieved by inoculation with isolated strains from the same site (1.2.5[Q](b) and 1.2.5[P+]). The question arises, therefore, whether substance(s) released into the inoculum from the crushed nodules affect nodulation. This possibility was examined in batch 4 by comparison of the infectivity and effectivity following inoculation of *A. rubra* with 1.2.5[Q](b) and crushed nodules separately, when diluted (crushed nodules only) and when mixed. The results of this experiment are shown in Tables 22 to 24. Nodulation was 100% with 1.2.5[Q](b) alone or when mixed with crushed nodule inoculum, and in both treatments the time for visible nodulation was 28 days, as observed previously in batch 1 (Table 19). However, when inoculation was with a crushed nodule preparation alone nodulation was delayed until 38 days. In this instance only 28% of plants were nodulated and this was reduced to 11% when the preparation was diluted 500 times. In batch 1 (Table 19) nodules took a similar length of time to develop on plants inoculated with crushed nodules but nodulation of test plants was much higher at 83%. With 1.2.5[Q](b) alone or mixed with a crushed nodule preparation, nodules were distributed over the upper 30% of the root system whereas when inoculation was with crushed nodules alone nodulation was limited to the root crown. The crushed nodules preparation therefore appeared to have no effect upon infection by 1.2.5[Q](b) and it seems unlikely that substance(s) present in the crushed nodule preparation inhibit nodulation. There was also little

Table 22: Interactions between crushed nodule inoculum and a *Frankia* strain isolated from those nodules: effects on the nodulation of inoculated *A. rubra* (batch 4)

Strain	Nodulation time (days)	Percentage nodulation	Nodule distribution[1]
1.2.5[Q](b)	28	100	2
1.2.5[Q](b) & crushed nodules	28	100	2
Crushed nodules	38	28	1
Crushed nodules diluted 1:500	38	11	1

[1] Nodule distribution is coded as follows: 1, confined to root crown and 2, confined to upper 30% of root system.

Table 23: Interactions between crushed nodule inoculum and a *Frankia* strain isolated from those nodules: effects on the growth of inoculated *A.rubra* (Batch 4)

Strain	Plant d.w.(g)	Nodule d.w.(g)	Nodule number	Ratio root to shoot d.w.	Plant / nodule d.w.
1.2.5[Q](b)	3.5	0.073	17 a	0.22 a	47.7
1.2.5[Q](b) and crushed nodules	3.9	0.099	35 b	0.29 a	41.9
Crushed nodules	5.3	0.125	6 a	0.43 b	39.4
Crushed nodules diluted 1:500	0.6	0.036	3	0.69	17.0

Nodules produced by inoculation with 1,2,5[Q](b) and 1,2,5[Q](b) plus crushed nodules were sp^-, Nodules produced by crushed nodule inoculum were sp^+/sp^-, Growth parameter values for 1,2,5[Q](b) and 1,2,5[Q](b) and crushed nodules nodules are means of 12 replicates, Data for crushed nodules are means of 7 replicates and for crushed nodules diluted 1;500 are means of 2 replicates, Means in the same column followed by the same letter are not significantly different ($p < 0,050$) from one another using Duncan's Multiple Range Test, Crushed nodules diluted 1;500 treatment was eliminated from any statistical analyses due to the low number of replicates, Mean plant dry weight values for non-inoculated control plants ranged from 0,03 to 0,09g, Analysis of Variance tables are shown in Table 23S, Appendix 4,

Table 24: Interactions between crushed nodule inoculum and a *Frankia* strain isolated from those nodules: effects on the nitrogen content of inoculated *A.rubra*. (Batch 4).

Strain	N(mg)g Plant d.w.$^{-1}$ [1]	Plant N (mg)[2]
1.2.5[Q](b)	23.40	81.09
1.2.5[Q](b) & crushed nodules	22.02	85.88
Crushed nodules	18.57	98.42
Crushed nodules diluted 1:500	18.57	11.14

[1] Figures are means of duplicate determinations.

[2] Calculated by multiplying the mean plant dry weight for each strain by the mean nitrogen content per gram plant dry weight.

effect on measured and calculated growth parameters (Table 23) and only nodule number per plant was changed significantly by the treatments; approximately twice the number of nodules were observed with 1.2.5[Q] plus crushed nodules compared to 1.2.5[Q] alone. In batch 1 the ratio of plant / nodule dry weight was significantly ($p < 0.050$) lower in plants inoculated with crushed nodules compared to those inoculated with 1.2.5[Q]. In batch 4 however, there were no significant effects of strain on this ratio. The differences between the two ratios in batch 1 were, however, small and as so many other factors could be involved it would be unwise to draw any definite conclusions. Inoculation with diluted crushed nodule preparation resulted in only 2 plants developing nodules and due to the resulting low number of replicates available they were excluded from any statistical analyses.

3.2.3 Comparison of effectivity of strains from *A. glutinosa* for nodulation and nitrogen fixation in *A. glutinosa* and *A. rubra*

Although the ability of strains to nodulate the host plant species was determined for all strains, detailed analysis of the effectivity of the strains for nodulation and nitrogen fixation was confined to growth studies of inoculated *A. rubra*. The effectivity of all the strains isolated from *A. glutinosa* was lower in the heterologous association than the most effective *A. rubra* strains in the homologous association (Table 20). Time did not permit assessment of the effectivity of all strains in association with *A. glutinosa*. However, in this experiment three strains isolated from *A. glutinosa*, and shown

previously to differ widely in their ability to nodulate and fix nitrogen in association with *A rubra*, were inoculated onto *A. glutinosa* in order to ascertain whether the same order of strain effectivity was maintained and whether the relative contributions of nodule specific activity and nodule growth to overall nodule effectivity was similar to that in the symbiosis with *A. rubra*.

The results are shown in Tables 25 and 26 with inoculated *A rubra* included for comparison. The results of previous experiments to ascertain the infectivity and effectivity of strains in heterologous association are shown in Tables 19 and 20. The results obtained on this occasion were broadly similar. Thus, nodules were visible on all inoculated *A. rubra* plants 21 days after inoculation and nodule distribution was as observed previously. There was a significant effect of strain on plant dry weight ($p < 0.010$) and a similar effect on plant nitrogen with the nitrogen content of plants inoculated with 1.1.8[Bu] between 5 and 6 times those inoculated with either 1.1.4[F] or 1.1.5[F] (Table 26). These differences were also accompanied by significant effects of strain on the ratios of plant / nodule dry weight ($p < 0.001$) with the ratio for the association with 1.1.8[Bu] between 2 and 3 times greater than with 1.1.4[F] and 1.1.5[F]; similar effects on ratios of plant nitrogen / nodule dry weight indicate that as in the previous comparison (batch 2) nodules formed with 1.1.8[Bu] were more effective in nitrogen fixation than with the other strains. The significantly different ($p < 0.050$) ratios of root to shoot dry weight observed in *A. rubra* inoculated with 1.1.8[Bu] compared to inoculation with either of the other two strains were not encountered in the previous comparison of the strains in batch 2.

Table 25: Growth of *A. rubra* and *A. glutinosa* inoculated with *Frankia* strains isolated from *A. glutinosa* nodules (Batch 5).

Strain	Plant d.w.(g)	Nodule d.w.(g)	Nodule number	Ratio root to shoot d.w.	Ratio plant to nodule d.w.
A. rubra					
1.1.8[Bu]	1.2 a	0.030	18	0.16 a	37.8 a
1.1.4[F]	0.4 b	0.025	12	0.22 b	15.6 b
1.1.5[F]	0.3 b	0.023	16	0.25 b	13.9 b
S.E.	0.19	0.0047	1.9	0.19	2.29
A. glutinosa					
1.1.8[Bu]	2.9 a	0.057 ab	46 a	0.25 ab	51.3
1.1.4[F]	4.2 b	0.078 a	33 b	0.29 a	56.0
1.1.5[F]	2.4 a	0.038 b	28 b	0.22 b	63.5
S.E.	0.43	0.0084	3.8	0.019	3.72

All figures are means of 12 replicates. Means within each species and column followed by the same letter are not significantly different ($p < 0.050$) from one another using Duncan's Multiple Range Test. Where means are not followed by a letter Analysis of Variance showed no significant effect ($p < 0.050$) of strain on the parameter. S.E. indicates standard error of the treatment mean. Mean plant dry weight values for non-inoculated control plants ranged from 0.011 to 0.029g. Analysis of Variance tables are shown in Table 25S, Appendix 4.

Table 26: Nitrogen content of *A. rubra* and *A. glutinosa* inoculated with *Frankia* strains isolated from *A. glutinosa* nodules (Batch 5).

Strain	N(mg)g plant d.w.$^{-1}$ [1]	Plant N (mg)[2]	Plant N / nodule d.w.
A. rubra			
1.1.8[Bu]	24.14	28.73	0.96
1.1.4[F]	16.55	6.45	0.26
1.1.5[F]	14.79	5.03	0.22
A. glutinosa			
1.1.8[Bu]	19.58	56.00	0.98
1.1.4[F]	18.55	76.98	0.99
1.1.5[F]	21.48	50.91	1.34

[1] Figures are means of duplicate determinations.

[2] Calculated by multiplying the mean plant dry weight for each strain by the mean nitrogen content per gram plant dry weight.

However, the relative effectivity of the symbiosis of the 3 strains with *A glutinosa* was quite different from their symbiosis with *A. rubra*. Thus, in contrast to the heterologous association there were no significant effects of strain on plant / nodule dry weight ratios (an indicator of nodule specific activity in nitrogen fixation since, as noted previously, plant dry weight in plants grown in combined nitrogen free conditions shows a highly significant correlation with plant nitrogen content; Figure 7). The differences in plant dry weight, therefore, must have arisen from differences in the dry weight of nodules produced per plant with the three strains, a situation similar to that found in previous assessments of the symbiosis of *A.rubra* with homologous strains in batches 1 to 3. Maximum plant dry weight and plant nitrogen, and therefore, greatest strain effectivity, were observed in *A. glutinosa* plants inoculated with 1.1.4[F], whereas in the heterologous association the effectivity of 1.1.8[Bu] was approximately three to four times that of the other strains. Moreover, there were no significant differences in nodule specific activity in the homologous associations. In the homologous associations there were large differences in nodule dry weight and, in general, increases in plant dry weight and plant nitrogen were accompanied by increases in nodule growth. In heterologous associations, however, nodule dry weights per plant were more constant and differences in plant dry weight or plant nitrogen were largely due to differences in nodule specific activity. These results, therefore, confirm the observations made in batches 1 to 3. It is notable that 1.1.8[Bu] formed associations with both *A. glutinosa* and *A. rubra* in which nodule specific activities were broadly comparable. The

heterologous symbioses with the other 2 strains tested gave rise to nodules in which the specific activity of nitrogen fixation was much lower than in their homologous associations.

3.2.4 Comparison of effectivity of strains isolated from *A.rubra* for nodulation and nitrogen fixation of *A.rubra* from different provenances

The results of batches 1 to 5 were obtained using fruits from one provenance of *A.rubra*. The experiment in batch 6 examined the effect of 3 *Frankia* strains on the growth of plants raised from fruits of 3 different provenances of *A.rubra* in order to determine whether there are provenance effects on plant/strain interactions. All the *Frankia* strains used in this experiment had been compared previously in batch 3.

The data of Tables 27 to 29 show the nodulation, growth and nitrogen content of *A.rubra* from 3 different provenances inoculated with 3 different *Frankia* strains. In all cases 100% of inoculated plants were nodulated at harvest. Nodulation in all strain/provenance combinations took 14 days with the exception of ArI4/McNab's Farm where it took 21 days. The distribution of nodules ranged from restriction to the root crown in ArI4 inoculated plants to distribution over the upper 30% of the root system in plants inoculated with either 1.2.19[Q] or 1.2.23[Q]. There was no relationship between provenance and nodule distribution, which was similar to previous comparisons of Lennox *A rubra* inoculated with strains in batch 3.

Table 27: Relationships between seed provenance, *Frankia* strain and nodulation and nitrogen fixation in *A. rubra*.

Seed Provenance and Strain	Nodulation time (days)	Percentage nodulation	Nodule distribution[1]
McNab's Farm			
ArI4	21	100	1
1.2.19[Q]	14	100	2
1.2.23[Q](b)	14	100	2
Menzies Bay			
ArI4	14	100	1
1.2.19[Q]	14	100	2
1.2.23[Q](b)	14	100	2
Prince Rupert			
ArI4	14	100	1
1.2.19[Q]	14	100	2
1.2.23[Q](b)	14	100	2

[1] Nodule distribution is coded as follows: 1, confined to root crown and 2, confined to upper 30% of root system.

Table 28: Growth of *A. rubra* from a number of provenances after inoculation with *Frankia* strains (Batch 6).

Provenance and Strain	Plant d.w.(g)	Nodule d.w.(g)	Nodule number	Ratio root to shoot d.w.	Plant / nodule d.w.
McNab's Farm					
ArI4	2.2	0.047	14	0.16 a	48.1
1.2.19[Q]	2.6	0.064	12	0.22 ab	46.0
1.2.23[Q](b)	2.4	0.049	11	0.25 b	51.7
S.E.	0.33	0.0088	1.2	0.029	4.10
Menzies Bay					
ArI4	2.4	0.045 a	8 a	0.21	53.9 a
1.2.19[Q]	2.7	0.041 a	9 a	0.16	65.1 b
1.2.23[Q](b)	3.5	0.069 b	15 b	0.21	48.0 a
S.E.	0.49	0.0080	1.0	0.022	3.46
Prince Rupert					
ArI4	2.0	0.041 a	12	0.18	59.8
1.2.19[Q]	1.7	0.032 a	12	0.15	53.6
1.2.23[Q](b)	2.8	0.055 b	11	0.18	51.1
S.E.	0.31	0.0059	1.4	0.014	7.51

Figures are means of 12 replicates. Means followed by the same letter are not significantly different ($p < 0.050$) from one another using Duncan's Multiple Range Test. Where means are not followed by a letter Analysis of Variance showed no significant effect ($p < 0.050$) of strain on the parameter. Two factor Analysis of Variance shows significant ($p < 0.010$) strain/provenance interaction in nodule number and significant ($p < 0.050$) strain/provenance interaction in the ratio of root to shoot dry weight. S.E. indicates standard error of the treatment mean. Mean plant dry weight values for non-inoculated control plants ranged from 0.010 to 0.020g. Analysis of Variance tables are shown in Table 28S.

Table 29: Nitrogen content of *A. rubra* from a number of seed sources after inoculation with *Frankia* strains (Batch 6).

Seed Provenance and Strain	N(mg)g plant d.w.$^{-1}$ [1]	Plant N (mg)[2]	Plant N / nodule d.w.
McNab's Farm			
ArI4	17.60	37.84	0.81
1.2.19[Q]	18.58	47.94	0.75
1.2.23[Q](b)	17.13	40.94	0.84
Menzies Bay			
ArI4	19.46	45.73	1.02
1.2.19[Q]	20.48	54.68	1.33
1.2.23[Q](b)	17.16	59.20	0.86
Prince Rupert			
ArI4	19.21	38.80	0.95
1.2.19[Q]	20.16	34.07	1.07
1.2.23[Q](b)	20.52	56.84	1.03

[1] Figures are means of duplicate determinations.

[2] Calculated by multiplying the mean plant dry weight for each strain by the mean nitrogen content per gram plant dry weight.

Significant effects of strain on plant dry weight were not observed for any of the provenances. Significant ($p < 0.050$) effects of strain on inoculated plants from McNab's Farm provenance were limited to small differences in the ratios of root to shoot dry weight; ArI4 and 1.2.23[Q] inoculated plants possessing lower and higher ratios respectively. Such different ratios may merely reflect differences in plant size as demonstrated by Evans (1972). However, in Menzies Bay provenance both nodule dry weight and nodule number were significantly ($p < 0.050$) higher in plants inoculated with 1.2.23[Q](b); in other strain/provenance combinations no such differences were observed. Differences were also observed in the nodule specific activity ratio (plant / nodule dry weight) in this provenance where plants inoculated with 1.2.19[Q] had a significantly ($p < 0.050$) larger ratio than those inoculated with either ArI4 or 1.2.23[Q]. Plant nitrogen followed the same trends and nodule specific activity was 20-35% greater with 1.2.19[Q] than with the other strains. Plants of Prince Rupert provenance inoculated with 1.2.23[Q] had a significantly ($p < 0.050$) higher nodule dry weight than those inoculated with ArI4 or 1.2.19[Q]. As nodule specific activity was similar for all strains tested in this provenance, this difference in nodulation resulted in a 40-60% higher plant dry weight, which was almost significant, and a higher plant nitrogen content.

In McNab's Farm provenance/strain combinations the nitrogen content of plant material was generally lower than in either of the other provenances. Although it is not possible to make any statistical comparisons and, therefore, any conclusions must be drawn cautiously the ratios of plant nitrogen / nodule dry weight suggest that nodule

specific activity may be slightly lower in this provenance. However, dry matter production per plant for the assimilation of a given amount of nitrogen is slightly higher in this provenance compared to associations with *A. rubra* of the Menzies Bay and Prince Rupert provenances.

In summary, the results show that *Frankia* strains do interact differently with different provenances of *A.rubra* although this is not necessarily apparent in differences in plant weight. The main effects were a significant strain/provenance interaction in nodule number ($p < 0.010$) and in root to shoot ratio ($p < 0.050$). However, in the previous assessment of the symbiotic performance of the strains in this batch (batch 3) with the Lennox provenance of *A. rubra*, nodule specific activity (plant / nodule dry weight) in plants inoculated with ArI4 was significantly ($p < 0.050$) greater than either 1.2.19[Q] or 1.2.23[Q](b) and effectivity, measured by plant growth, of both ArI4 and 1.2.23[Q](b) was significantly ($p < 0.050$) greater than that of 1.2.19[Q] (Table 20). These results suggest that there may be a greater interaction of strain with some provenances than was shown in the particular combinations studied in batch 6.

3.2.5 Nitrogenase activity, hydrogen evolution and hydrogen uptake

Assays of nitrogenase activity, hydrogen evolution and hydrogen uptake were performed on nodules harvested from plants in batches 1 to 5 to determine whether there were any relationships between these parameters and plant growth and nitrogen fixation with different *Frankia* strains. A preliminary examination of the time course of

acetylene reduction, hydrogen evolution and of hydrogen uptake by nodules from plants inoculated with ArI4 (grown and assayed under identical experimental conditions to those of batches 1 to 5) showed that activities of acetylene reduction and hydrogen evolution fell steadily and continually over the period of assay, whilst hydrogen uptake remained relatively constant but was extremely variable (Figure 12). The rates of decline of both nitrogenase activity and hydrogen evolution were similar over long periods; with rates of acetylene reduction at 30, 60, 90 and 120 minutes after the start of incubation 82, 65, 62 and 61% and H_2 evolution 89, 93, 68 and 62% respectively of the rate during the first 5 minutes. Over the first 60 minutes of incubation, therefore, the rate of decline in H_2 evolution was less than that of acetylene reduction. Furthermore, H_2 evolution was measured before acetylene reduction. Both of these factors will tend to reduce the calculated values for relative efficiency (Table 30). However, because the amount of H_2 evolved is so small relative to acetylene reduced the effect will be almost negligible.

Tables 30 and 31 show measured rates of acetylene reduction, hydrogen evolution, and calculated values of relative efficiency (R.E.); H_2 uptake and ratios of hydrogen uptake to nitrogenase activity for nodules harvested from plants grown in batches 1 to 5. Hydrogen evolution by all nodules was small in comparison to nitrogenase activity and the R.E. of nodules ranged from 0.98 to 1.00. All strains were, therefore, highly efficient and it is unlikely that differences in R.E. contributed to the observed differences in nodule specific activity. Since measurements of hydrogen evolution and uptake were made over the first 30 minutes of incubation whereas measurements of

Figure 12: A) Nitrogenase activity, B) H_2 evolution and C) H_2 uptake of nodules over time.

Measurements are of nodules attached to root incubated at 25°C.

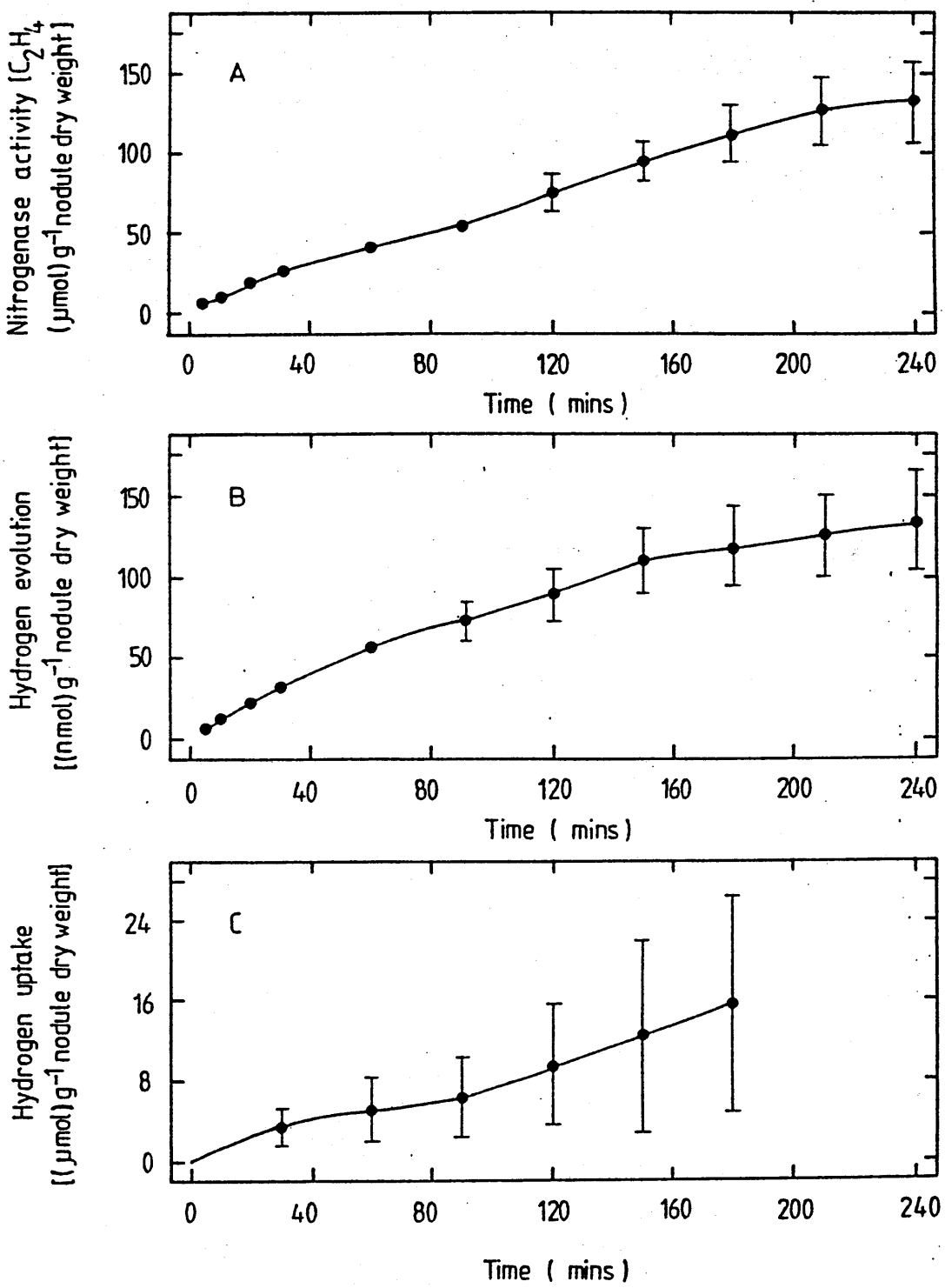

A
Nitrogenase activity [C_2H_4 (μmol) g^{-1} nodule dry weight]
150
100
50
0
0
40
80
120
160
200
240
Time (mins)
B
Hydrogen evolution [(nmol) g^{-1} nodule dry weight]
150
100
50
0
0
40
80
120
160
200
240
Time (mins)
C
Hydrogen uptake [(μmol) g^{-1} nodule dry weight]
24
16
8
0
0
40
80
120
160
200
240
Time (mins)

Table 30: Nitrogenase activity and hydrogen evolution in nodules produced by inoculation of *Alnus* spp. with *Frankia*.

Species/batch /strain	H_2 evolved[1] (nmol)$h^{-1}g^{-1}$ nodule f.w. ± S.E	N_2ase activity [C_2H_4 evolved(nmol) $h^{-1}g^{-1}$ nodule f.w.] ± S.E.	Relative efficiency ±S.E.
A. rubra			
Batch 1			
ArI4	12 ± 0	9148 ± 1975	1.00 ± 0
1.2.5[Q](b)	22 ± 13	13108 ± 7842	1.00 ± 0.001
ArI5	44 ± 8	11332 ± 1933	1.00 ± 0.001
1.2.5[P+]	13 ± 5	9387 ± 3465	1.00 ± 0
ArI3	26 ± 7	6843 ± 542	1.00 ± 0.001
Crushed nodules	25 ± 9	9609 ± 1831	1.00 ± 0.001
1.1.1[BuC]	111 ± 44	17561 ± 2983	0.99 ± 0.002
Batch 2			
ArI4	22 ± 11	4172 ± 2068	1.00 ± 0.001
1.1.8[Bu]	9 ± 2	3914 ± 1460	1.00 ± 0.001
Agn1C12	41 ± 13	2492 ± 1372	0.98 ± 0.008
1.1.2[Q]	44 ± 6	15469 ± 1361	1.00 ± 0
1.1.4[F]	2 ± 1	1741 ± 786	1.00 ± 0
1.1.5[F]	8 ± 3	3770 ± 710	1.00 ± 0.001
Batch 3			
ArI4	14 ± 3	7154 ± 1672	1.00 ± 0
1.2.23[Q](b)	7 ± 5	2990 ± 1837	1.00 ± 0.001
1.1.14[Q]	1 ± 0	677 ± 434	0.99 ± 0.003
1.2.20[Bu]	3 ± 1	4412 ± 655	1.00 ± 0
1.2.13[Q]	12 ± 8	10085 ± 8066	1.00 ± 0.001
1.2.13[Bu]	4 ± 1	3536 ± 969	1.00 ± 0
1.2.19[Q]	14 ± 1	8463 ± 553	1.00 ± 0
1.2.15[Bu]	13 ± 6	10309 ± 5256	1.00 ± 0

Table 30 continued:

Species/batch /strain	H_2 evolved[1] $(nmol)h^{-1}g^{-1}$ nodule f.w. ± S.E	N_2ase activity [C_2H_4 evolved(nmol) $h^{-1}g^{-1}$ nodule f.w.] ± S.E.	Relative efficiency ±S.E.
Batch 4			
1.2.5[Q](b)	19 ± 5	9706 ± 2645	1.00 ± 0
1.2.5[Q](b) & crushed nodules	53 ± 23	5519 ± 1456	0.98 ± 0.007
Batch 5			
1.1.8[Bu]	118 ± 32	15132 ± 3600	0.99 ± 0.001
1.1.4[F]	23 ± 8	2900 ± 1209	0.99 ± 0.003
1.1.5[F]	37 ± 9	3584 ± 727	0.99 ± 0.001
A. glutinosa			
Batch 5			
1.1.8[Bu]	38 ± 10	13714 ± 2078	1.00 ± 0.001
1.1.4[F]	3 ± 1	3956 ± 408	0.99 ± 0.003
1.1.5[F]	15 ± 11	6270 ± 3479	0.98 ± 0.006

Figures are means of 3 replicates.
[1] Calculated over period of 0 to 30 minutes of incubation.
[2] Calculated over period 65 to 95 minutes of incubation.

Table 31: Nitrogenase activity and hydrogen uptake in nodules produced by inoculation of *Alnus* spp. with *Frankia*.

Species/batch /strain	H_2 uptake[1] (µmol) h^{-1} g^{-1} nodule f.w. ± S.E	N_2ase activity [C_2H_4 evolved (µmol) h^{-1} g^{-1} nodule f.w.] ± S.E.	Ratio of H_2 uptake to N_2ase activity ± S.E.
Batch 1			
ArI4	2.3 ± 1.11	8.4 ± 4.44	8.2 ± 5.63
1.2.5[Q](b)	16.9 ± 2.66	16.8 ± 4.46	1.0 ± 0.15
ArI5	19.7 ± 6.47	7.8 ± 1.23	0.5 ± 0.16
1.2.5[P+]	18.2 ± 15.30	12.2 ± 3.12	1.9 ± 1.35
ArI3	5.8 ± 2.21	11.9 ± 2.36	1.9 ± 0.09
1.1.1[BuC]	8.1 ± 4.31	9.4 ± 2.54	1.1 ± 0.09
Batch 2			
ArI4	3.5 ± 0.94	3.7 ± 1.82	1.0 ± 0.31
1.1.8[Bu]	8.0 ± 3.14	2.1 ± 0.58	0.3 ± 0.008
Agn1C12	6.7 ± 3.37	3.6 ± 0.53	2.3 ± 1.96
1.1.2[Q]	17.5 ± 8.74	1.4 ± 0.42	0.1 ± 0.009
1.1.4[F]	2.9 ± 1.44	2.3 ± 1.35	1.6 ± 1.58
1.1.5[F]	7.5 ± 5.46	2.1 ± 0.51	0.7 ± 0.53
Batch 3			
ArI4	1.9 ± 1.26	4.7 ± 0.08	5.5 ± 2.23
1.1.14[Q]	21.2 ± 6.12	6.1 ± 3.30	0.5 ± 0.39
1.2.20[Bu]	8.8 ± 5.16	3.2 ± 0.74	0.5 ± 0.14
1.2.13[Q]	4.6 ± 3.17	3.5 ± 0.43	1.6 ± 1.14
1.2.13[Bu]	ND	3.3 ± 1.13	ND
1.2.19[Q]	3.0 ± 1.89	3.7 ± 1.90	3.5 ± 3.25
1.2.5[Bu]	12.5 ± 6.34	1.6 ± 0.45	0.2 ± 0.17

Table 31 continued:

Species/batch /strain	H_2 uptake[1] (μmol) h^{-1} g^{-1} nodule f.w. ± S.E	N_2ase activity $^{5C}_2H_4$ evolved (μmol) h^{-1} g^{-1} nodule f.w.] ± S.E.	Ratio of H_2 uptake to N_2ase activity ± S.E.
Batch 4			
1.2.5[Q](b)	10.2 ± 3.38	10.7 ± 2.85	1.2 ± 0.25
1.2.5[Q](b) & crushed nodules	9.1 ± 5.88	11.4 ± 5.62	1.8 ± 0.29
Crushed nodules	2.2 ± 0.92	1.7 ± 0.69	2.3 ± 1.92

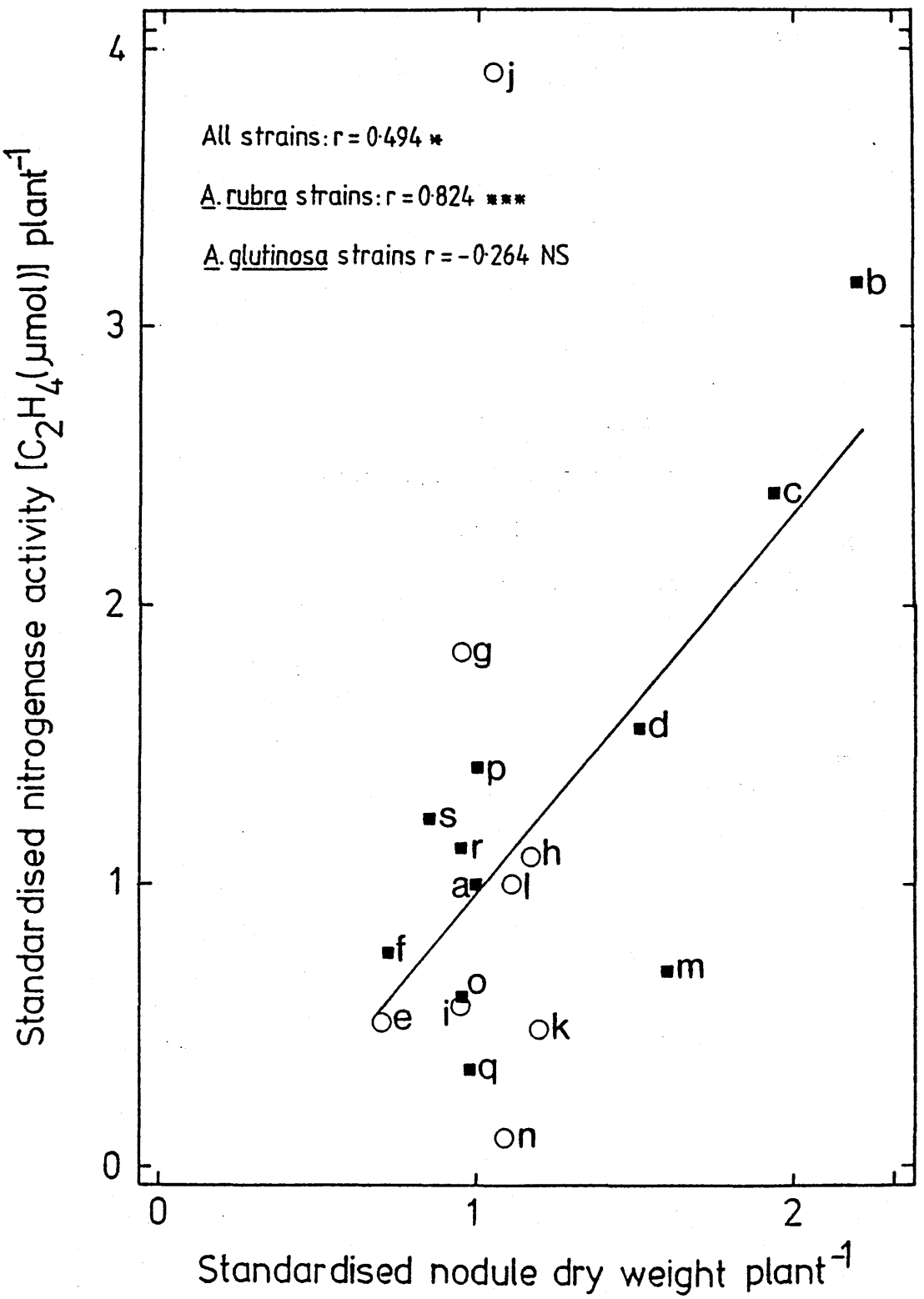
Figures are means of 3 replicates.
[1] Calculated over period of 0 to 30 minutes of incubation.
[2] Calculated over period 65 to 95 minutes of incubation.
ND Not determined.

nitrogenase activity were only made over the period 65 to 95 minutes after incubation had begun, it is likely that values of R.E. would actually have been higher than those calculated as would the ratios of nitrogenase activity to hydrogen uptake. The R.E. of nodules produced as the result of inoculation with ArI4 was constant between batches in which it was included. Hydrogen uptake was shown by all nodules but was highly variable as, in consequence, were the calculated ratios of hydrogen uptake to nitrogenase activity. The large standard errors and the variability between batches in nodules produced by inoculation with ArI4 make it difficult to identify any trends in the data.

Some researchers have used the nitrogenase assay, carried out at one time in the period of plant growth, to assess strain effectivity. The data obtained in the present study permitted assessment of the validity of this approach for the *A. rubra Frankia* symbioses studied. The relationship between plant nitrogenase activity and nodule dry weight in plants harvested in batches 1 to 3 is shown in Figure 13; all measurements are standardised for the performance of ArI4 in each batch. Comparisons of Figure 9 where effectivity was determined by nitrogen content and Figure 13 where effectivity was estimated by acetylene reduction of nodules show it is possible to observe the same general relationship between strains, with a significant correlation ($p < 0.050$) between the measures in homologous associations and no significant correlation in heterologous associations. This demonstrates again that in the strains examined nodule growth was the major determinant of differences in strain effectivity in many homologous associations whereas nodule specific activity was the major determinant in heterologous associations. However, in many cases the

Figure 13: Relationship between plant nitrogenase activity and plant nodule dry weight of *A. rubra* inoculated with *Frankia* in batches 1 to 3 standardised relative to growth/activity of ArI4 inoculated plants.

(■) indicates strains isolated from *A. rubra* (○) indicates strains isolated from *A. glutinosa*. Strain letters as in the legend to Figure 7(p.115).


All strains: r = 0·494 *
A. rubra strains: r = 0·824 ***
A. glutinosa strains r = -0·264 NS
Standardised nitrogenase activity [C_2H_4(μmol)] plant^{-1}
Standardised nodule dry weight plant^{-1}
0
1
2
3
4
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
s

order of nodule specific activity between different strains was changed considerably using the different methods of assessment. Particularly striking, for example, are the differences in specific activity between plants inoculated with strains 1.1.1[BuC] and 1.1.14[Q] with an almost complete reversal in the relative activities of the nodules using the different methods. The ratio of plant nitrogen / nodule dry weight is, as stated previously, a measure of nodule specific activity in nitrogen fixation. As demonstrated, there is usually a good correlation between this ratio and the ratio plant / nodule dry weight in plants relying solely on symbiotic nitrogen fixation for growth. Figures 14 and 15 show specific nitrogenase activity plotted against the ratio of plant / nodule dry weight and plant nitrogen / nodule dry weight respectively for plants grown in batches 1 to 3 all standardised relative to the performance of ArI4 in each case. As expected from the changed order of specific activity in Figure 13 there is a significant ($p < 0.050$) negative correlation between nitrogenase activity and plant nitrogen / nodule dry weight (Figure 15), indicating that as nodule specific activity increased specific nitrogenase activity fell. There was, however, no correlation between specific nitrogenase activity and plant / nodule dry weight, since the correlation coefficient, r, was not quite significant. However, the same general trend can be observed as in the plot of specific nitrogenase activity against plant nitrogen / nodule dry weight. Clearly, therefore, although the two methods for determining strain effectivity did identify similar trends great caution must be exercised in the interpretation of results obtained using single point estimates of nitrogenase activity as an indicator of symbiotic performance of different strains.

Figure 14: Relationship between nitrogenase activity and specific activity (plant/nodule dry weight of *A. rubra* inoculated with *Frankia* strains in batches 1 to 3 standardised relative to growth/activity of ArI4 inoculated plants.

(■) indicates strains isolated from *A. rubra* (○) indicates strains isolated from *A. glutinosa*. Strain letters as in the legend to Figure 7(p.115).

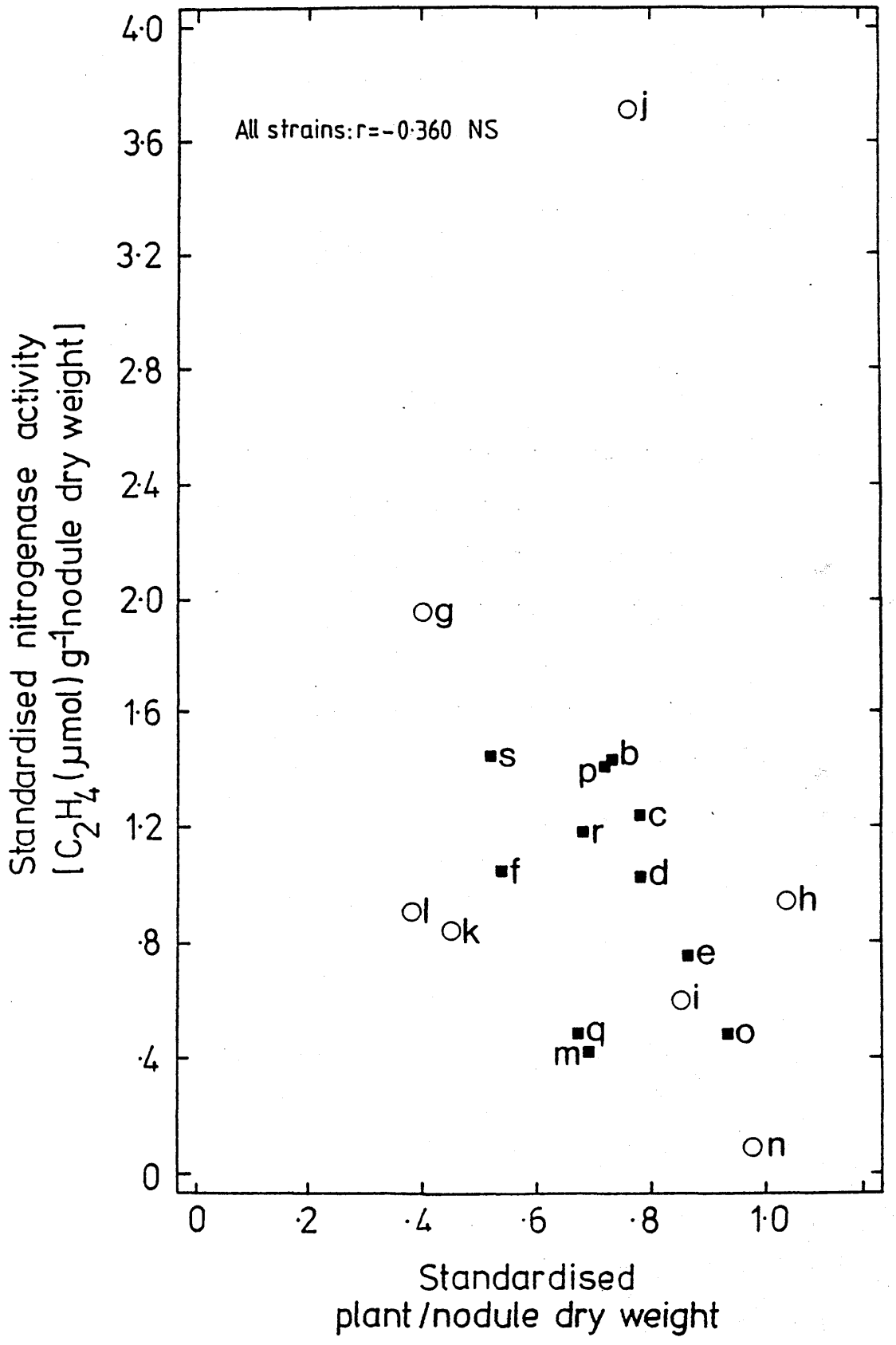
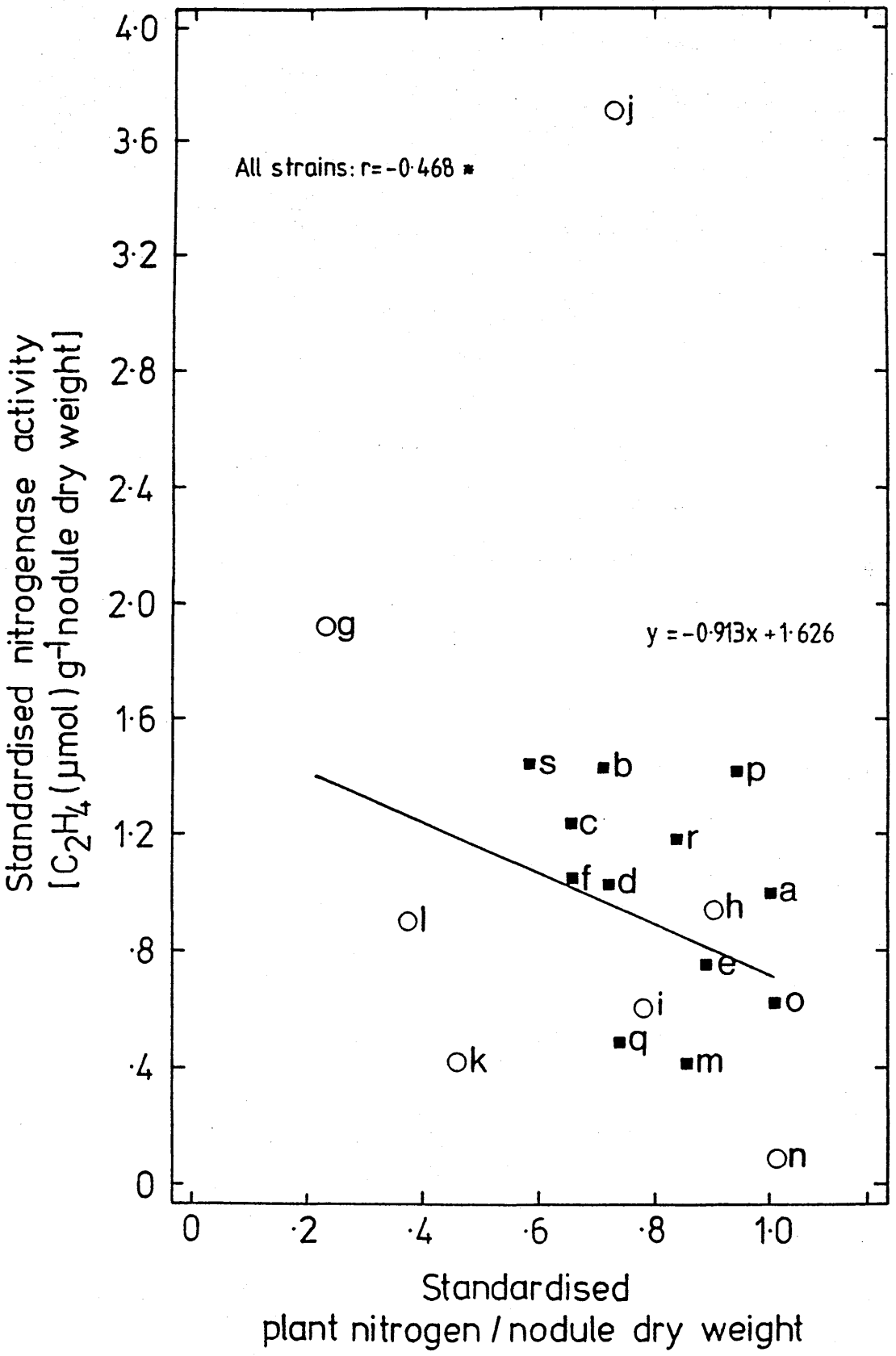

All strains: r=-0·360 NS
Standardised nitrogenase activity
[C_2H_4 (μmol) g^{-1} nodule dry weight]
Standardised
plant/nodule dry weight
4·0
3·6
3·2
2·8
2·4
2·0
1·6
1·2
·8
·4
0
·2
·4
·6
·8
1·0
j
g
s
p
b
r
c
f
d
h
l
k
e
i
q
m
o
n

Figure 15: Relationship between nitrogenase activity and specific activity (plant/nodule dry weight)of plants inoculated with *Frankia* strains in batches 1 to 3 standardised relative to growth/activity of ArI4 inoculated plants.

(■) indicates strains isolated from *A. rubra* (○) indicates strains isolated from *A. glutinosa*. Strain letters as in the legend to Figure 7(p.115).


All strains: r=-0·468 *
y = -0·913x + 1·626
Standardised nitrogenase activity
[C2H4 (µmol) g-1 nodule dry weight]
Standardised
plant nitrogen / nodule dry weight
4·0
3·6
3·2
2·8
2·4
2·0
1·6
1·2
·8
·4
0
·2
·6
1·0
j
g
s
b
p
c
r
f
d
a
h
l
e
i
o
q
m
k
n

3.3.0 Utilisation of *Frankia* for inoculation of Alnus in field plantings

3.3.1 Nodulation of *A. rubra* in the field by indigenous *Frankia*

An assessment of nodulation and growth of *A.rubra* growing on a number of sites of different soil type in Scotland and Northern England is shown in Table 32. On peat sites, nodulation was poor and localised and plant growth was also relatively poor whereas on sites with a mineral soil nodulation was good, nodules were distributed throughout the root system and plant growth was also relatively good. Soils at the survey sites were all acidic with pH in the range 3.2 to 3.8. There was no obvious relationship between soil pH or site exposure and either nodulation or growth of *A.rubra*. While peats generally were particularly deleterious to both nodulation and growth there was variation between sites and on Shin 45, a more established site, nodulation and growth was not limited so severely as on the other peat soils.

The potential of soils collected from each of these sites (although away from the *Alnus* planting) for nodulation of both *A. rubra* and *A. glutinosa* was examined further in glasshouse tests by inoculation of soils with *Frankia* (Table 33). None of the soil samples infected either species prior to inoculation and it could be assumed, therefore, that either endophyte was not present or conditions were not suitable for infection. Subsequently, after inoculating with *Frankia* all soils supported nodulation of host seedlings thus

Table 32: Nodulation and growth of *A. rubra* on a number of field plantings.

Site[1]	Characteristics	pH	Plantation age(years)	Nodulation	Performance of *A.rubra*
Shin 45	Peat, highly organic	3.6	12	Limited but some spread from crown	Moderate, some dieback
Shin 65	Peat, highly organic	3.8	9	Extremely poor and restricted to crown	Extremely poor, many dead
Shin 93	Peat, highly organic	3.6	3	Extremely poor and restricted to crown	Moderate to poor
Rumster 9	Peat, highly organic	3.5	13	Extremely poor and restricted to crown	Very poor
S.Yorks 9	Discontinuous iron pan, low organic content	3.5	13	Good	Good
Wykeham 116	Iron pan, low organic content	3.2	14	Good	Excellent, ready for harvest

[1] Further details of sites and characteristics Table 1.

Table 33: Nodulation potential of soils for *A. rubra* and *A. glutinosa*.

Site[1]	Characteristics	pH	% nodulation before inoculation		% nodulation after inoculation[2]	
			A.rubra	*A.glutinosa*	*A.rubra*	*A.glutinosa*
Shin 45	Peat, highly organic.	3.6	0	0	80	82
Shin 65	Peat, highly organic	3.8	0	0	42	100
Shin 93	Peat, highly organic	3.6	0	0	84	91
Rumster 9	Peat, highly organic	3.5	0	0	42	61
S. Yorks 9	Discontinuous iron pan, low organic content	3.5	0	0	50	89
Wykeham 116	Iron pan, low organic content	3.2	0	0	45	55
Control	Perlite	6.0	0	0	100	100

[1] Further details of sites and characteristics Table 1.
[2] Soil inoculated with strain 1.1.1[BuC] and strain 1.2.5[Q](b) on separate occasions.

confirming that it was lack of endophyte which prevented nodulation. However, differences in the percentage of plants nodulated in the different soils suggest again that soil characteristics additional to pH are important for nodulation and fixation.

3.2.2 Glasshouse experiments to investigate interactions between *Frankia*, host plant species, provenance of *A. rubra* and soil type

In this experiment the relative effects, on the growth of *A. glutinosa* and *A. rubra* (two provenances), of soil type and inoculation with different sources of *Frankia* were assessed.

The low pH of the peat was one of the major differences between the soils (Table 34). The fibrous structure of the peat also was different from the particulate brown earths. The main difference in the chemical composition of the 3 soils was in the carbon content, which was similar for each of the brown earths but 7 times higher in the peat. Peat contained only about one third and one fifth of the phosphate content of the Leadburn and Elibank brown earths respectively. Available phosphate in the Elibank soil was about twice that in the Leadburn brown earth. The brown earth from Leadburn also contained less available potassium than the other two soils. The levels of these nutrients were low in relation to the requirements for plant growth and deficiency effects were alleviated during the course of the experiment by addition of K_2HPO_4. Available magnesium in the peat was up to 5 times that of the other soils whilst available calcium was highest in the Leadburn soil. The total nitrogen content

Table 34: Physical and Chemical Properties of the Irradiated Soils.

	Soil		
	Elibank	Leadburn	Peat
Physical Properties			
Soil Type	Brown Earth	Brown Earth	Peat
pH	4.1	4.5	2.9
%Sand	60	62	NA
%Silt	29	23	NA
%Clay	11	16	NA
Chemical Properties			
%C	7	7	49
%N	0.44	0.36	1.01
$\%PO_4$	0.1	0.06	0.02
Extractable Elements (mg $100g^{-1}$)[1]			
PO_4	2.4	1.3	NA
K	18	13	24
Ca	29	65	44
Mg	7.4	9.3	56

[1] Extractable in neutral ammonium acetate.
NA Not available.

of all the soils was low but that of peat was more than twice that of the Elibank and Leadburn soils. However, as only total nitrogen was measured the likely effects of these differences on plant growth are not known.

All surviving inoculated plants were nodulated at harvest with the exception of some plants grown in peat where only 70% were infected. These non-nodulated plants grown in peat were chlorotic and grew extremely poorly; many had died, presumably due to poor nutrition. A number of plants grown in soils from Elibank and Leadburn also died, not from nutritional causes but due to infestation with a vine weevil, *Otiorrhyncus sulcatus*; these plants were included in the data analyses as missing values. Although some control plants became nodulated during the course of the experiment this was generally only at a low level and nodules were limited to the periphery of the root system. This suggests that infections occurred late in the growth period and, therefore, contributed little to differences in the growth of plants.

The most significant effects were of soil type on plant dry weight and nitrogen accretion. Both dry weight and nitrogen accretion were least in peat; at harvest, dry weight and nitrogen accretion were up to 46 times greater for *A. rubra* on Leadburn and more than 80 times greater for *A. rubra* on Elibank soil. Differences in the growth of *A. glutinosa* on the 3 soils were generally less than for *A. rubra*, with growth on Leadburn only up to 3 to 4 times greater, and on Elibank only approximately 7 times greater than on peat (Tables 35 to 37, Figures 16 and 17).

Figure 16: Histogram of stem (shaded area) or plant (non-shaded area) nitrogen of *Alnus* growing in soils from A, Elibank; B, Leadburn and C, peat after inoculation with crushed nodules from 1, control - no inoculum; 2, ArI5; 3, Corvallis; 4, Lennox; 5, Milngavie. The letters X,Y,Z indicate the plant genotype; *A. glutinosa*, *A. rubra* (Terrace provenance) and *A. rubra* (Timberlands provenance) respectively.

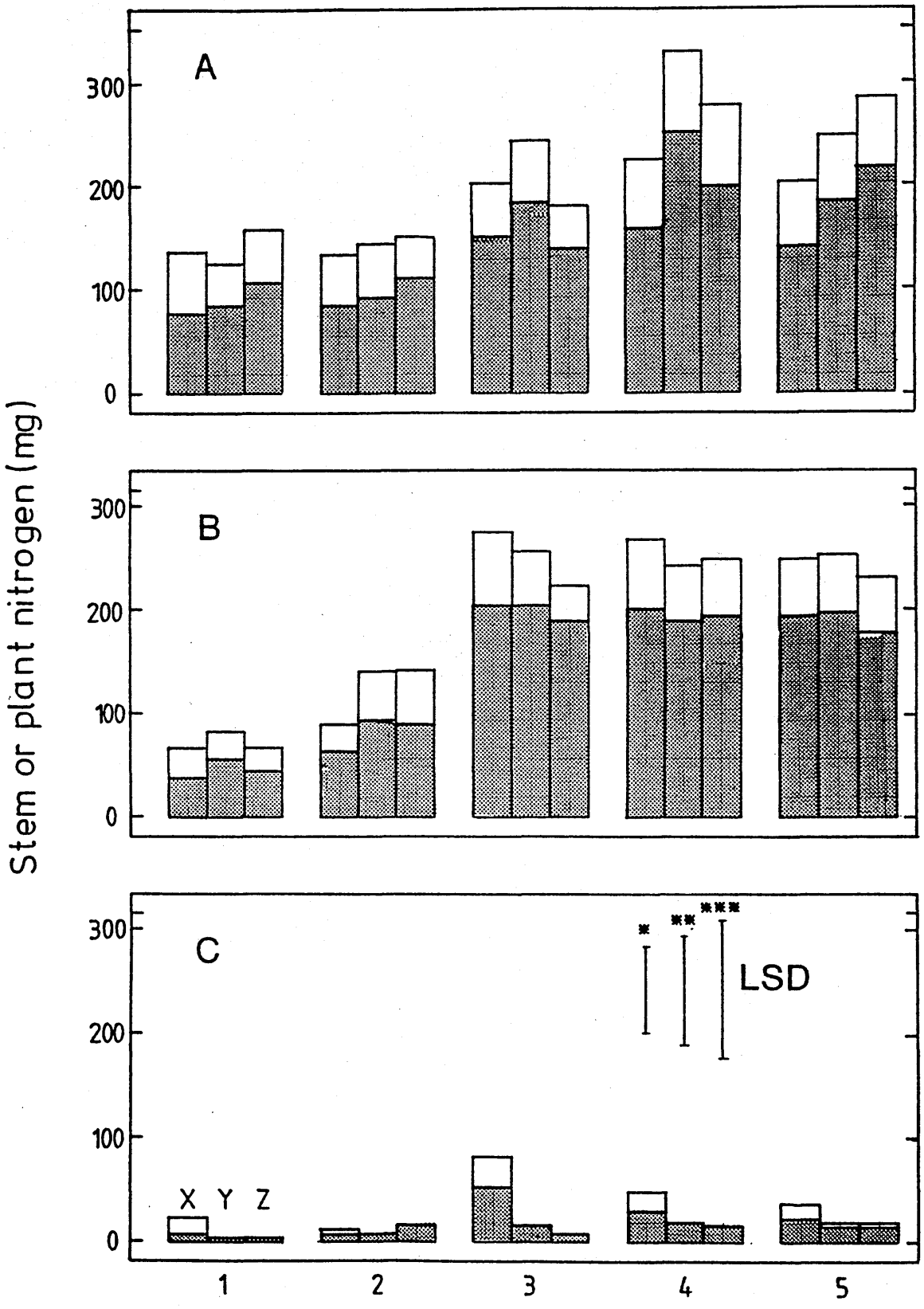

A
B
C
300
200
100
0
Stem or plant nitrogen (mg)
X Y Z
*
**
***
LSD
1
2
3
4
5

Figure 17: Histogram of plant dry weight of *Alnus* - see legend to Figure 16 for details.

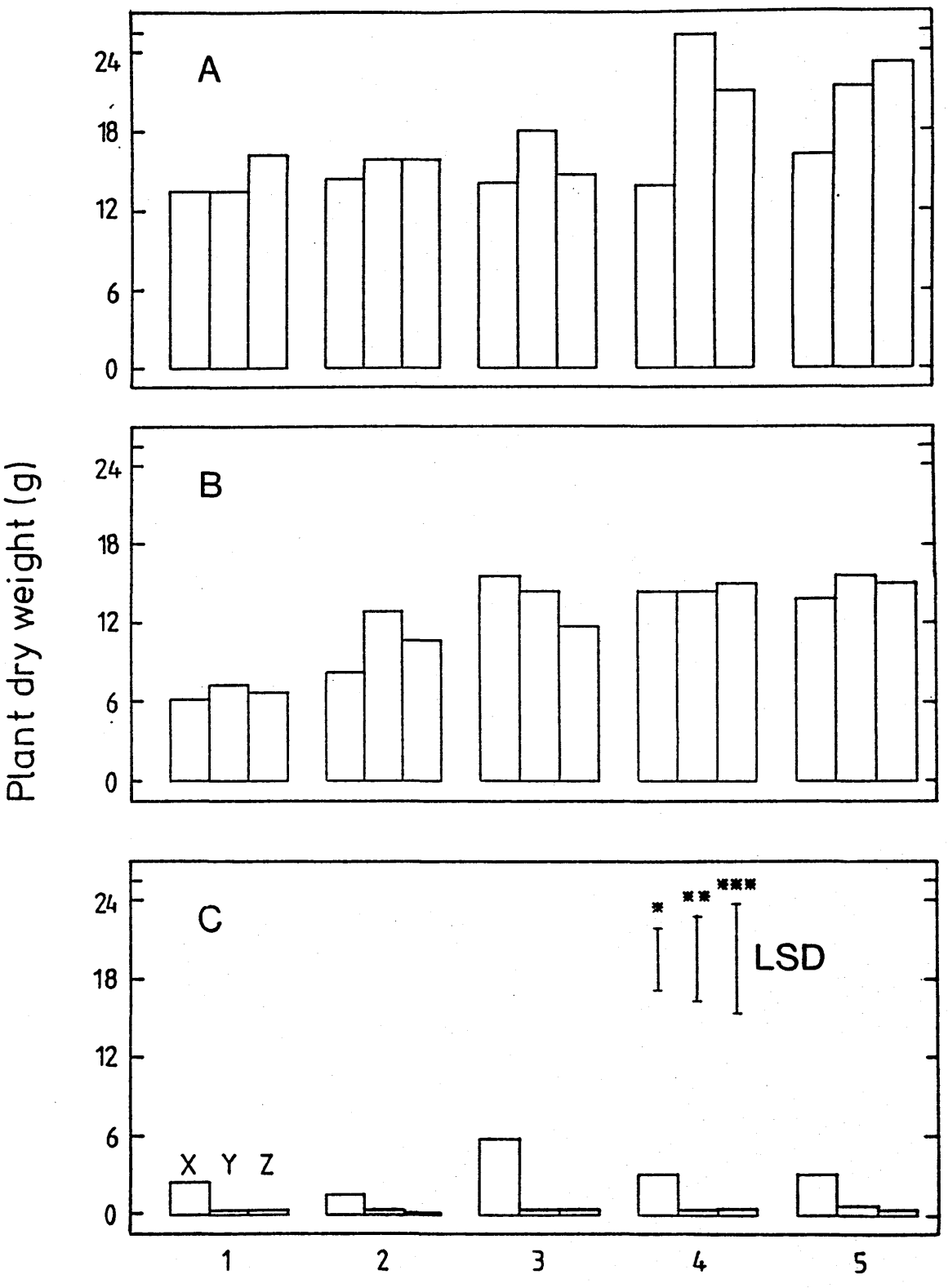

Plant dry weight (g)
A
B
C
24
18
12
6
0
X Y Z
LSD
1
2
3
4
5

Table 35: Mean nitrogen content (mg) per plant of *Alnus* inoculated with *Frankia* from different sources and grown in different soils.

Soil	Inoculum	Host Plant[1] *A. glutinosa*	*A. rubra* (Terrace)	A. rubra (Timberlands)
Elibank	Control	138.1	127.4	161.2
	ArI5	135.5	146.0	152.2
	Corvallis	202.9	246.1	182.3
	Lennox	226.7	332.2	278.1
	Milngavie	206.0	249.6	286.7
Leadburn	Control	66.7	80.9	66.6
	ArI5	90.6	140.0	140.8
	Corvallis	274.2	255.5	224.3
	Lennox	266.9	240.9	249.3
	Milngavie	248.7	252.2	230.5
Peat	Control	20.6	4.7	4.7
	ArI5	11.5	8.9	14.5
	Corvallis	81.9	16.2	8.2
	Lennox	46.9	20.4	16.7
	Milngavie	37.3	17.7	17.3

[1] Locations in brackets are of *A. rubra* provenances.

Table 36: Mean dry weights (g) of *Alnus* inoculated with *Frankia* from different sources and grown in different soils.

Soil	Inoculum	Host Plant[1] *A. glutinosa*	*A. rubra* (Terrace)	*A. rubra* (Timberlands)
Elibank	Control	13.63	13.66	16.16
	ArI5	14.28	15.82	15.88
	Corvalis	14.08	18.13	14.65
	Lennox	13.73	25.08	21.02
	Milngavie	16.22	21.40	22.99
Leadburn	Control	6.23	7.38	6.72
	ArI5	8.23	12.85	10.66
	Corvalis	15.65	14.43	11.80
	Lennox	14.41	14.82	14.98
	Milngavie	13.84	15.64	15.15
Peat	Control	2.36	0.16	0.31
	ArI5	1.61	0.31	0.08
	Corvalis	5.67	0.30	0.23
	Lennox	3.08	0.27	0.29
	Milngavie	2.90	0.57	0.43
		$p<0.050$	$p<0.010$	$p<0.001$
LSD : all levels		2.956	3.885	4.963
: same level		2.497	3.282	4.193
SED : all levels		1.508		
: same level		1.274		

[1] Locations in brackets are of *A. rubra* provenances.

Table 37: Analysis of Variance of dry weights of *Alnus* inoculated with *Frankia* from different sources and grown in different soils.

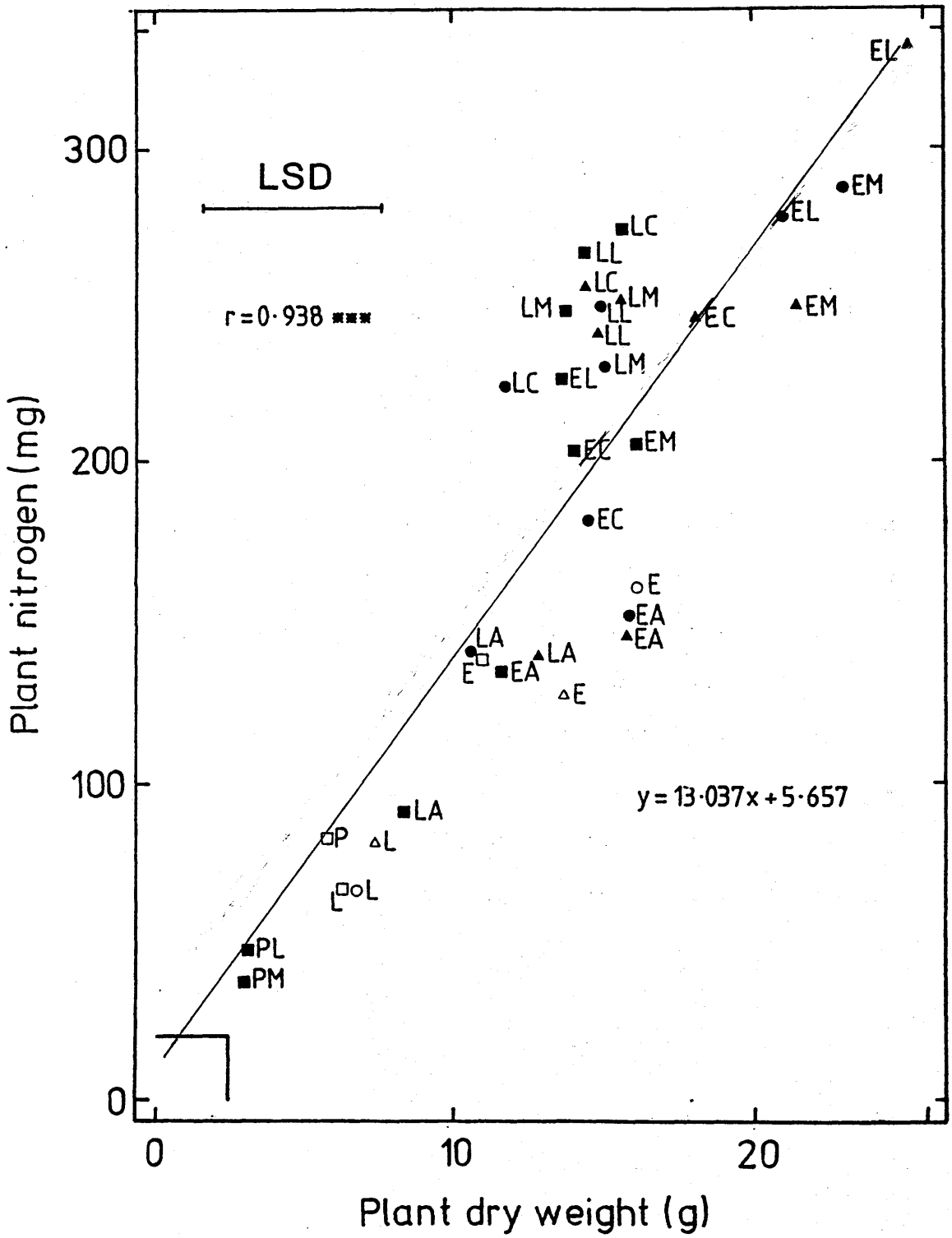
DUE TO	DF(MV)	SS	SS%	MS	F	
Block Stratum	4	1166.80	2.18	291.70		
Block.Soil Stratum						
Soil	2	39633.39	74.04	19816.70	174.192	***
Error	8	910.11	1.70	113.76		
Total	10	40543.50	75.74	4054.35		
Block.Soil.Units Stratum						
Inoculum	4	2986.18	5.58	746.54	45.964	***
Host	2	151.21	0.28	75.60	4.655	**
Soil.Inoculum	8	1800.35	3.36	225.04	13.856	***
Soil.Host	4	1628.35	3.04	407.09	25.064	***
Inoculum.Host	8	597.35	1.12	74.67	4.597	***
Soil.Inoculum.Host	16	836.07	1.56	52.25	3.217	***
Error	707(136)	11483.13	21.45	21.45	16.24	
Total	749	19482.63	36.40	26.01		
Grand Total	763	61192.92	114.32			

Inoculation with *Frankia* in most cases significantly increased dry weight accretion. A major exception was in plants inoculated with ArI5 and grown in Elibank soil where no significant effect on dry weight was observed. Although, in general, there was a good relationship between plant nitrogen and plant dry weight (Figure 18) the percentage nitrogen content of uninoculated plants and of plants inoculated with ArI5 was lower than that of plants inoculated with other *Frankia* by up to 50%. Thus, although differences in dry weight due to inoculation with different *Frankia* of plants grown in Elibank soil were either small or not significant, differences in nitrogen accretion were much more marked. For example, dry matter accretion in *A. rubra* (Terrace) plants grown in Elibank soil and inoculated with Corvallis *Frankia* was not significantly different from plants inoculated with ArI5. However, the nitrogen content of the former plants was 75% higher than the ArI5 inoculated plants (Tables 35 and 38). In general, the effects of inoculation were much less marked on plants grown in the Elibank soil than on plants grown in the other soils. Differences in nitrogen content of inoculated plants were up to 4 times greater than non-inoculated controls in the peat and Leadburn soils but only 2 times greater in the Elibank soil.

Plant genotype also affected plant growth, with significant effects of plant species on plant dry weight and evidence of interactions with soil type and *Frankia* source (Tables 36 and 37; Figure 16). The largest observed effect of plant genotype was on Elibank soil between *A. rubra* (Terrace) and *A. glutinosa*. Also of interest is the generally higher plant dry weight of *A. glutinosa* growing in peat, although the difference is only significant in plants inoculated with

Figure 18: Relationship between plant nitrogen and plant dry weight of plants grown in different soils after inoculation with different sources of crushed nodules.

First letter indicates the soil type; L, Leadburn; P, peat and E, Elibank. Second letter indicates source of inoculum. A, ArI5; C, Corvallis; L, Lennox; M, Milngavie. The symbols ■, ● and ▲ indicate *A. glutinosa, A. rubra* (Timberlands provenance) and *A. rubra* (Terrace provenance) respectively. □ , ○ and △ indicate corresponding non-inoculated controls. Data within box is for *A. rubra* grown in peat unless otherwise indicated.


LSD
r = 0·938 ***
y = 13·037x + 5·657
Plant nitrogen (mg)
Plant dry weight (g)
0
100
200
300
0
10
20
EL
EM
EL
LC
LL
LC
LM
LM
LL
LL
EC
EM
LM
LC
EL
EC
EM
EC
E
EA
EA
LA
E
EA
LA
E
LA
P
L
L
L
PL
PM

Frankia from Corvallis. Although in some treatments there were no differences in dry weight accretion between *A. glutinosa* and *A. rubra*, for example in plants grown in Leadburn soil and inoculated with *Frankia* from Lennox, in other combinations differences were highly significant. *A rubra* grown in Elibank soil and inoculated with Lennox *Frankia*, for example, had approximately twice the dry weight accretion of *A. glutinosa*. Differences between provenances were less notable but were apparent in some cases. For example, the better growth of *A. rubra* (Terrace) when compared to *A. rubra* (Timberlands) grown on Elibank soil and inoculated with Corvallis *Frankia*.

Comparison of shoot nitrogen, however, showed no significant genotype effects (Tables 38 and 39). This discrepancy when either plant dry weight or nitrogen content were used to compare growth may be a result of 'blocking' of shoot nitrogen assessments which reduced the number of replicates per treatment and thus reduced the sensitivity of the ANOVA.

There were significant differences in nodule dry weight (Tables 40 and 41; Figure 19) and nodule number (Tables 42 and 43) between plants from different treatments. The correlation ($P < 0.001$) of nodule number with both plant dry weight and plant nitrogen shows that increase in plant size would seem to be associated with increased nodule numbers (Figures 20 and 21). The largest differences in nodule dry weight per plant were due to plant species, with plant nodule dry weight in all cases being less in *A. glutinosa* than *A. rubra*, except for plants grown in peat where the converse was true (Figure 19).

Table 38: Mean shoot nitrogen content (mg) of *Alnus* inoculated with *Frankia* from different sources and grown in different soils.

Soil	Inoculum	Host Plant[1] *A. glutinosa*	*A. rubra* (Terrace)	*A. rubra* (Timberlands)
Elibank	Control	78.6	84.9	109.6
	ArI5	85.5	93.3	112.7
	Corvallis	150.9	187.1	139.6
	Lennox	160.4	252.0	202.2
	Milngavie	140.8	185.9	218.3
Leadburn	Control	38.3	54.2	44.0
	ArI5	62.5	94.1	89.7
	Corvallis	204.3	204.7	188.2
	Lennox	201.0	189.3	194.1
	Milngavie	192.1	198.5	179.4
Peat	Control	7.3	3.9	3.2
	ArI5	7.9	7.8	14.3
	Corvallis	52.8	14.7	6.8
	Lennox	29.5	19.3	15.3
	Milngavie	21.5	14.9	14.9
		p<0.050	p<0.010	p<0.001
LSD :		39.298	51.649	65.985
SED :		20.050		

[1] Locations in brackets are of *A. rubra* provenances.

Table 39: Analysis of Variance of shoot nitrogen of *Alnus* inoculated with *Frankia* from different sources and grown in different soils.

DUE TO	DF(MV)	SS	SS%	MS	F	
Block Stratum	4	4420.4	3.42	1105.1		
Block.Units Stratum						
Soil	2	83181.0	64.34	41590.5	413.746	***
Host	2	492.0	0.38	246.0	2.447	NS
Inoculum	4	33371.0	25.81	8342.8	82.994	***
Soil.Host	4	1194.0	1.54	498.5	4.959	***
Soil.Inoculum	8	14364.8	11.11	1795.6	17.863	***
Host.Inoculum	8	1237.2	0.96	154.7	1.538	NS
Soil.Host.Inoculum	15(1)	2247.4	1.74	149.8	1.490	NS
Error	145(31)	14575.7	11.27	100.5		
Total	188	151462.9	117.16	805.7		
Grand Total	192	155883.3	120.58			

Figure 19: Histogram of nodule dry weight of *Alnus* - see legend to Figure 16 for details (p.165).

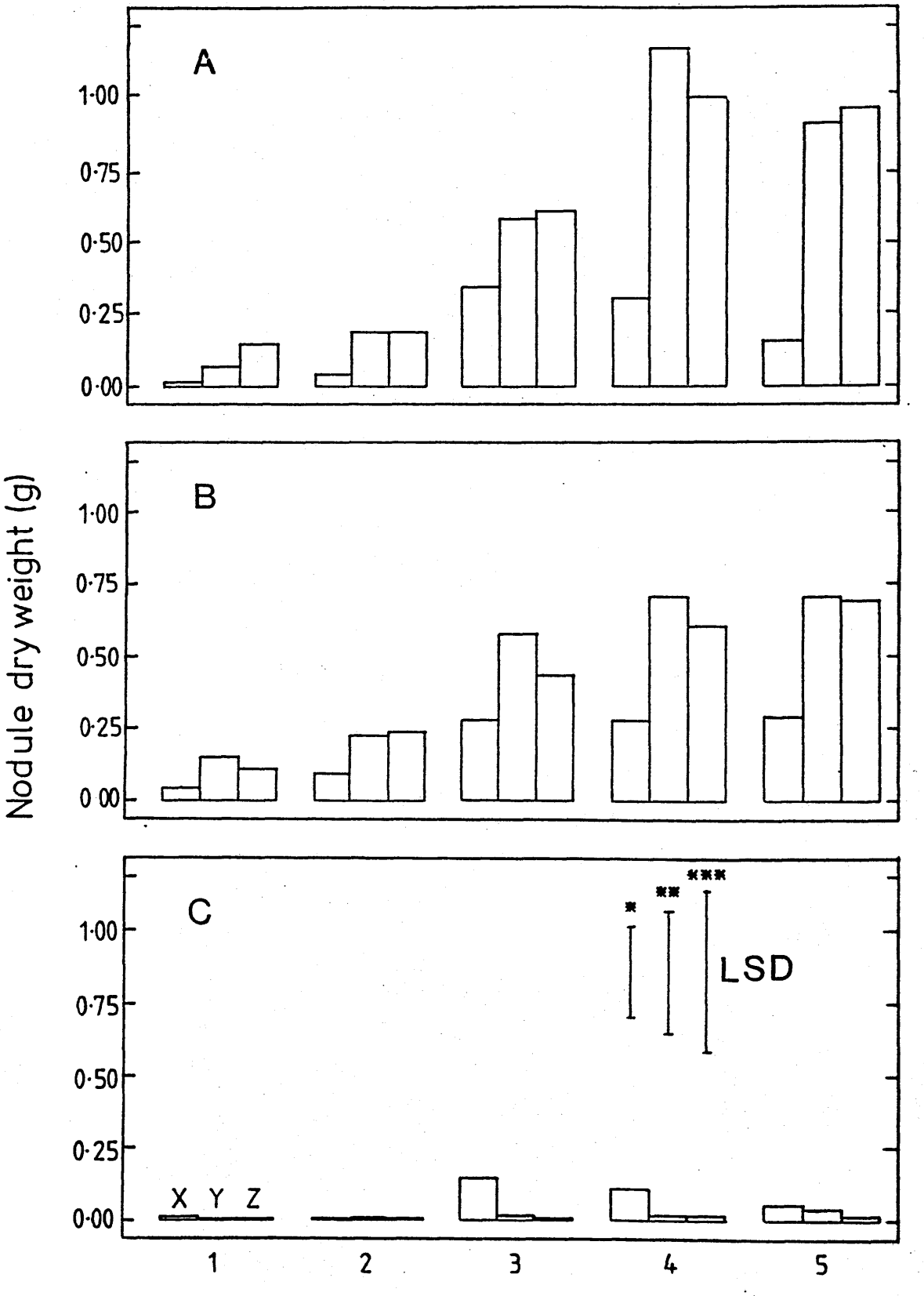

A
B
C
Nodule dry weight (g)
1·00
0·75
0·50
0·25
0·00
0 00
*
**
***
LSD
X Y Z
1
2
3
4
5

Table 40: Mean plant nodule dry weight (g) of *Alnus* inoculated with *Frankia* from different sources and grown in different soils.

Soil	Inoculum	Host Plant[1] *A. glutinosa*	*A. rubra* (Terrace)	*A. rubra* (Timberlands)
Elibank	Control	0.0183	0.0607	0.1468
	ArI5	0.0346	0.1845	0.1897
	Corvalis	0.3467	0.5853	0.6061
	Lennox	0.3062	1.1805	1.0101
	Milngavie	0.1614	0.9133	0.9708
Leadburn	Control	0.0383	0.1479	0.1088
	ArI5	0.0943	0.2315	0.2430
	Corvalis	0.2821	0.5876	0.4323
	Lennox	0.2759	0.7193	0.6129
	Milngavie	0.2872	0.7151	0.7021
Peat	Control	0.0093	0	-0.0003
	ArI5	0.0010	0.0022	0.0005
	Corvalis	0.1428	0.0107	0.0041
	Lennox	0.1006	0.0164	0.0081
	Milngavie	0.0576	0.0343	0.0073
		$p<0.050$	$p<0.010$	$p<0.001$
LSD : all levels		0.18144	0.23846	0.30465
: same level		0.15372	0.20204	0.25811
SED : all levels		0.09257		
: same level		0.07843		

[1] Locations in brackets are of *A. rubra* provenances.

Table 41: Analysis of Variance of plant nodule dry weight of *Alnus* inoculated with *Frankia* from different sources and grown in different soils.

DUE TO	DF(MV)	SS	SS%	MS	F	
Block Stratum	4	1.98555	1.65	0.49639		
Block.Soil Stratum						
Soil	2	29.92221	24.82	14.96111	35.265	***
Error	8	3.39400	2.82	0.42425		
Total	10	33.31619	27.64	3.33162		
Block.Soil.Units Stratum						
Inoculum	4	24.99361	20.74	6.24840	101.581	***
Host	2	8.40028	6.97	4.20014	68.282	***
Soil.Inoculum	8	12.32172	10.22	1.54021	25.039	***
Soil.Host	4	7.57833	6.29	1.89458	30.800	***
Inoculum.Host	8	4.29915	3.57	0.53739	8.736	***
Soil.Inoculum.Host	16	4.00585	3.32	0.25037	4.070	***
Error	707(136)	43.48862	36.08	0.06151		
Total	749	105.08752	87.18	0.14030		
Grand Total	763	140.38927	116.47			

Table 42: Mean nodule numbers on *Alnus* inoculated with *Frankia* from different sources and grown in different soils.

Soil	Inoculum	Host Plant[1] *A. glutinosa*	*A. rubra* (Terrace)	*A. rubra* (Timberlands)
Elibank	Control	1.3	0.8	1.9
	ArI5	1.1	3.8	8.5
	Corvallis	38.2	99.0	77.6
	Lennox	41.0	124.4	123.2
	Milngavie	10.3	125.0	81.8
Leadburn	Control	1.7	4.3	5.8
	ArI5	3.2	1.8	9.8
	Corvallis	71.9	115.9	90.3
	Lennox	109.8	143.4	147.2
	Milngavie	55.7	92.9	107.9
Peat	Control	0.7	0.0	-0.1
	ArI5	0.2	0.1	0.2
	Corvallis	9.1	1.1	1.3
	Lennox	25.7	2.9	1.0
	Milngavie	8.5	5.1	1.8
		$p<0.050$	$p<0.010$	$p<0.001$
LSD : all levels		27.40	36.01	46.01
: same level		24.58	32.30	41.27
SED : all levels		13.98		
: same level		12.54		

[1] Locations in brackets are of *A. rubra* provenances.

Table 43: Analysis of Variance of nodule numbers on *Alnus* inoculated with *Frankia* from different sources and grown in different soils.

DUE TO	DF(MV)	SS	SS%	MS	F	
Block Stratum	4	29283	0.99	7321		
Block.Soil Stratum						
Soil	2	591250	20.08	295625	40.462	***
Error	8	58450	1.99	7306		
Total	10	649700	22.07	64970		
Block.Soil.Units Stratum						
Inoculum	4	875012	29.72	218753	139.073	***
Host	2	88566	3.01	44283	28.153	***
Soil.Inoculum	8	373950	12.70	46744	29.717	***
Soil.Host	4	101783	3.46	25446	16.177	***
Inoculum.Host	8	64966	2.21	8121	5.163	***
Soil.Inoculum.Host	16	84365	2.87	5273	3.352	***
Error	702(141)	1104204	37.51	1573		
Total	744	2692843	91.47	3619		
Grand Total	758	3371826	114.54			

Figure 20: Relationship between plant dry weight and nodule number per plant of plants grown in different soils after inoculation with different sources of crushed nodules - see legend to Figure 18 for details of symbols and lettering(p.171).

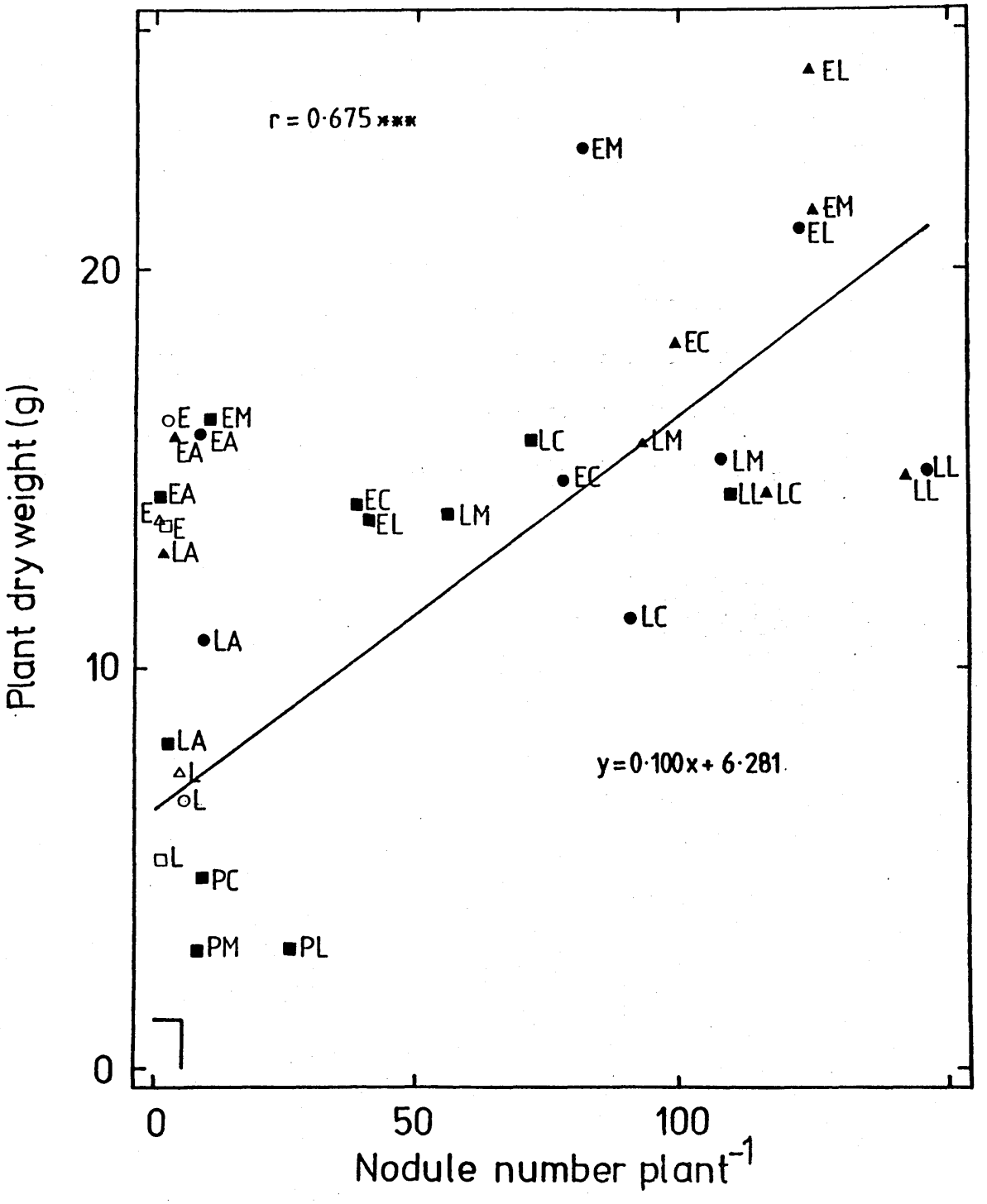
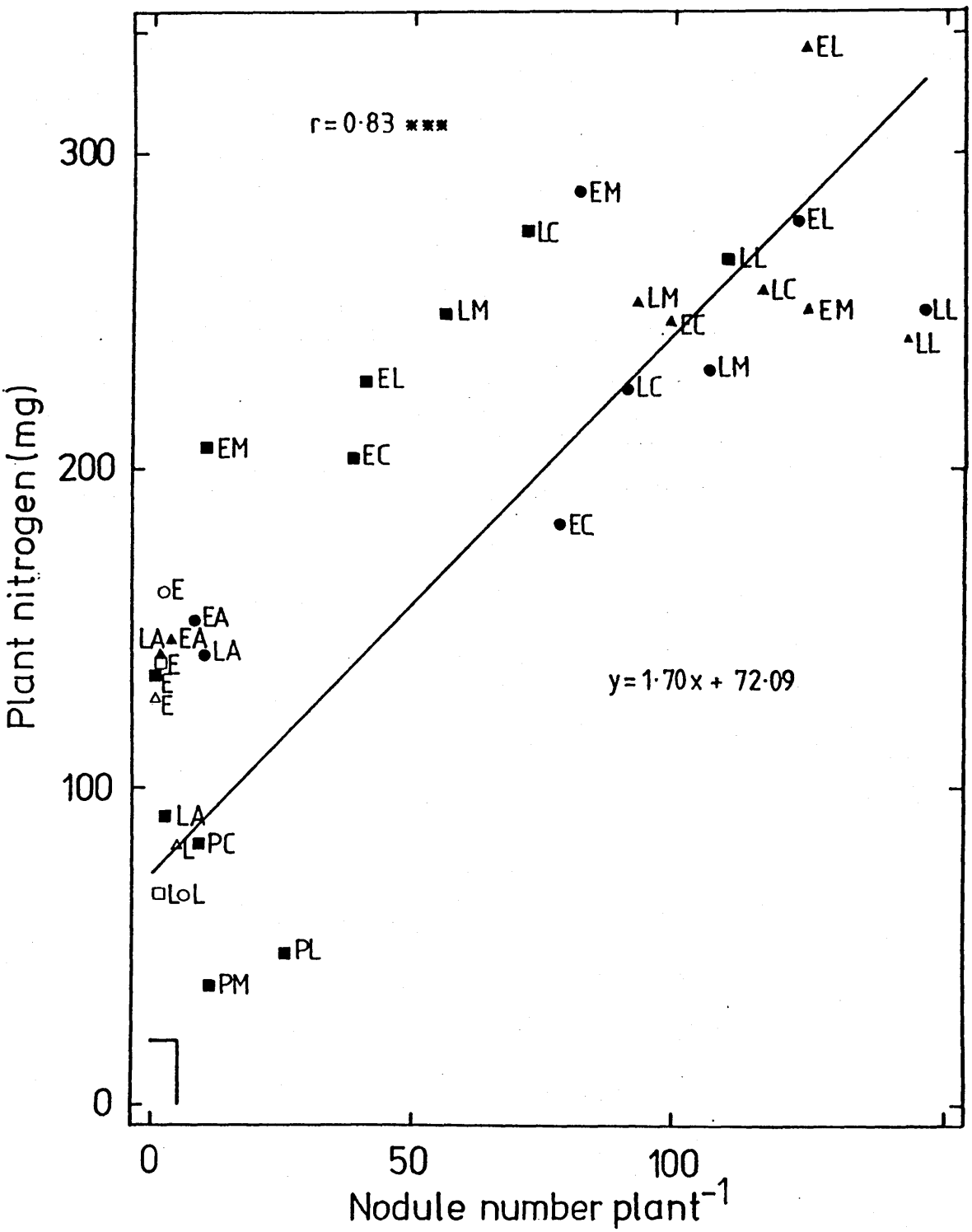

r = 0·675***
y = 0·100x + 6·281
Plant dry weight (g)
Nodule number plant^{-1}
0
10
20
0
50
100
EL
EM
EM
EL
EC
E
EM
EA
EA
LC
LM
LM
EC
LL
LL
EA
EC
EL
LM
LL
LC
E
E
LA
LC
LA
LA
L
L
L
PC
PM
PL

Figure 21: Relationship between plant nitrogen and nodule number per plant of plants grown in different soils after inoculation with different sources of crushed nodules - see legend to Figure 18 for details of symbols and lettering(p.171).


r = 0·83 ***
y = 1·70x + 72·09
Plant nitrogen (mg)
Nodule number plant^{-1}
0
100
200
300
0
50
100

Variations in plant dry weight, plant nitrogen and nodule dry weight were, therefore, due to differences in soil type, plant genotype, inoculation and source of *Frankia* and in some cases interaction between these factors. The overall effects on the symbioses are shown in Figures 22 and 23. Although the variation in the data makes it difficult to discern differences between specific treatments it is possible to observe a number of general relationships and groupings in the data. Soil type had a large effect on growth and, in general, the data from *A. rubra* grown in either peat, Leadburn or Elibank soils each form separate groups. Uninoculated control plants form a group and also, loosely associated with this group are plants inoculated with ArI5. It is clear that the poor growth of ArI5 inoculated plants was accompanied by a low nodule dry weight per plant. Inoculum also influenced groupings according to soil type. For example, plants inoculated with Corvallis *Frankia* and grown on Elibank soil generally had a lower nodule dry weight than plants inoculated with either Lennox or Milngavie *Frankia*. A close relationship between nodule growth and growth of *A. rubra* is demonstrated by the correlation of both plant dry weight (Figure 22) and plant nitrogen (Figure 23) with nodule dry weight ($p < 0.001$). However, in inoculated *A. glutinosa* which also formed separate groups, despite large differences in plant growth nodule dry weight per plant was similar. Although with *A. glutinosa* nodule dry weight correlated significantly with plant dry weight ($p < 0.050$) and plant nitrogen ($p < 0.001$) the regression line for symbioses with *A. glutinosa* was steeper than for symbioses with *A. rubra*. This suggests that in *A. rubra* differences in plant growth were more associated with differences in nodulation than in

Figure 22: Relationship between dry weight and nodule dry weight per plant of plants grown in different soils after inoculation with different sources of crushed nodules - see legend to Figure 18 for details of symbols and lettering(p.171).

Overlay: indicates general groupings of data.

A: Peat, *A. rubra*

B: Leadburn, *A. rubra* - except for those plants inoculated with ArI5; also Elibank, *A. rubra* inoculated with Corvallis (Timberlands provenance) and ArI5.

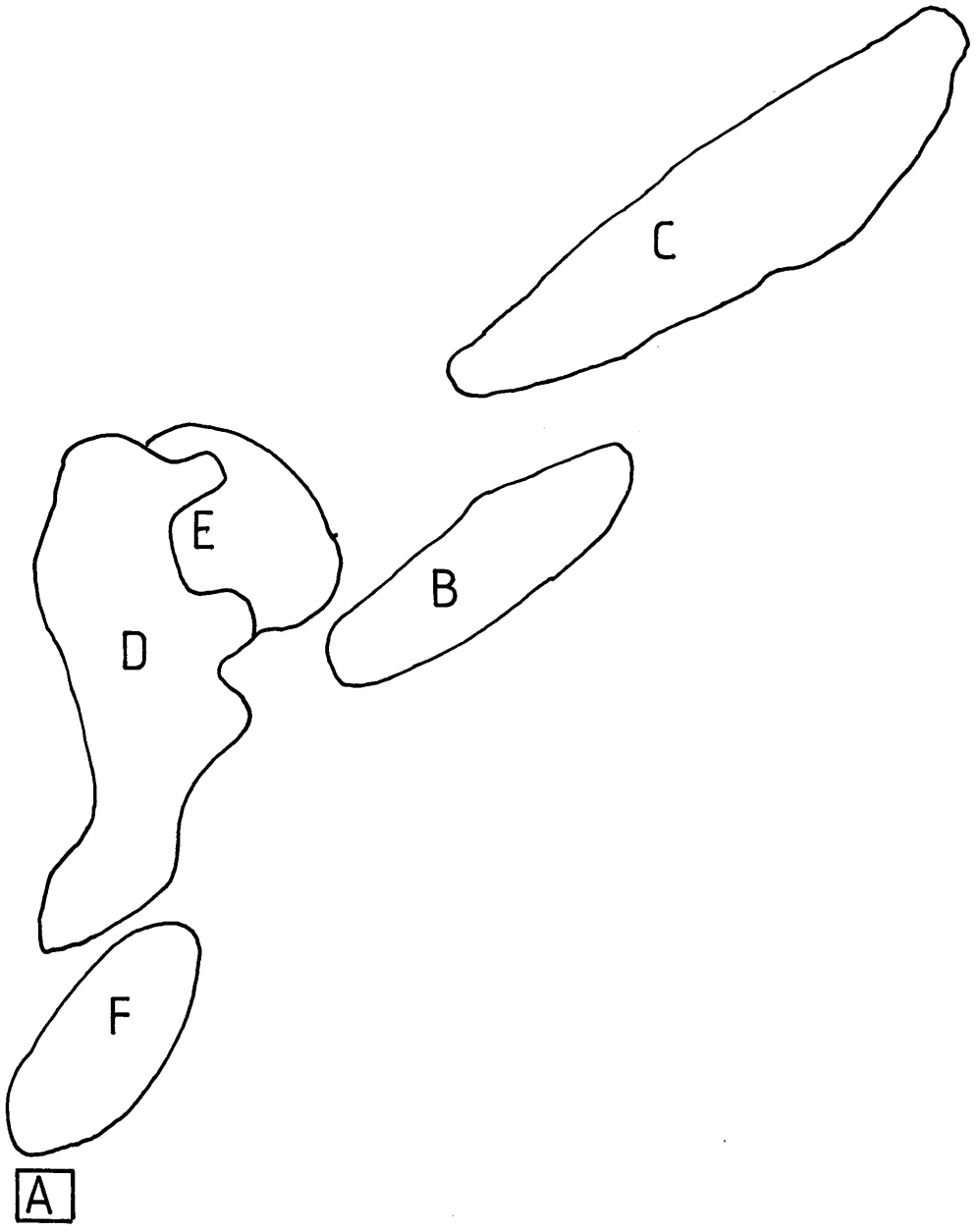
C: Elibank, *A. rubra* - except for those in group B.

D: Elibank and Leadburn, *A rubra* uninoculated.

E: Elibank and Leadburn *A glutinosa*.

F: Peat, *A. glutinosa*.

Overlay Figure 22



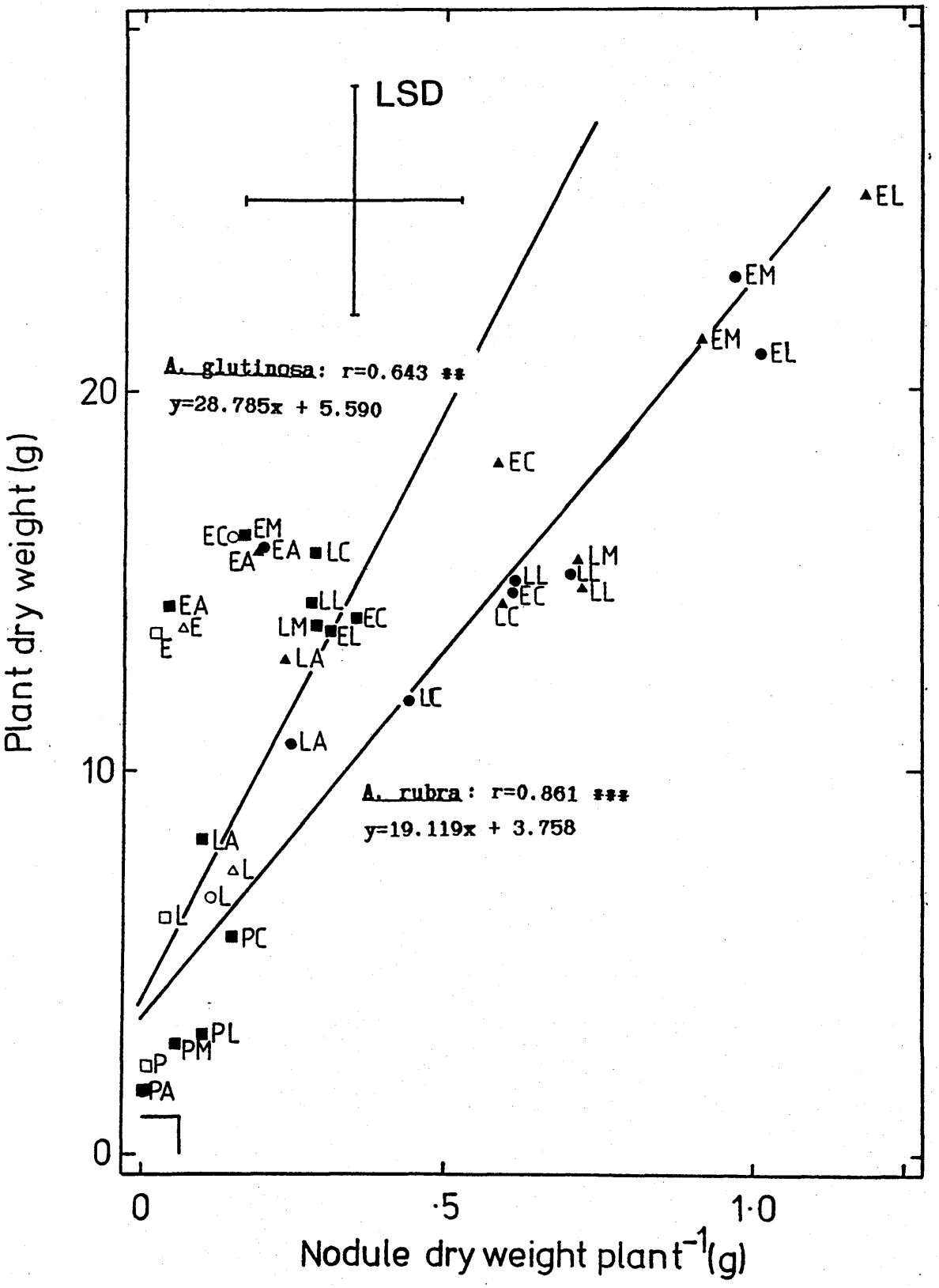

LSD
A. glutinosa: r=0.643 **
y=28.785x + 5.590
A. rubra: r=0.861 ***
y=19.119x + 3.758
Plant dry weight (g)
Nodule dry weight plant⁻¹(g)
0
10
20
0
·5
1·0

Figure 23: Relationship between plant nitrogen and nodule dry weight per plant of plants grown in different soils after inoculation with different sources of crushed nodules - see legend to Figure 18 for details of symbols and lettering (p.171).

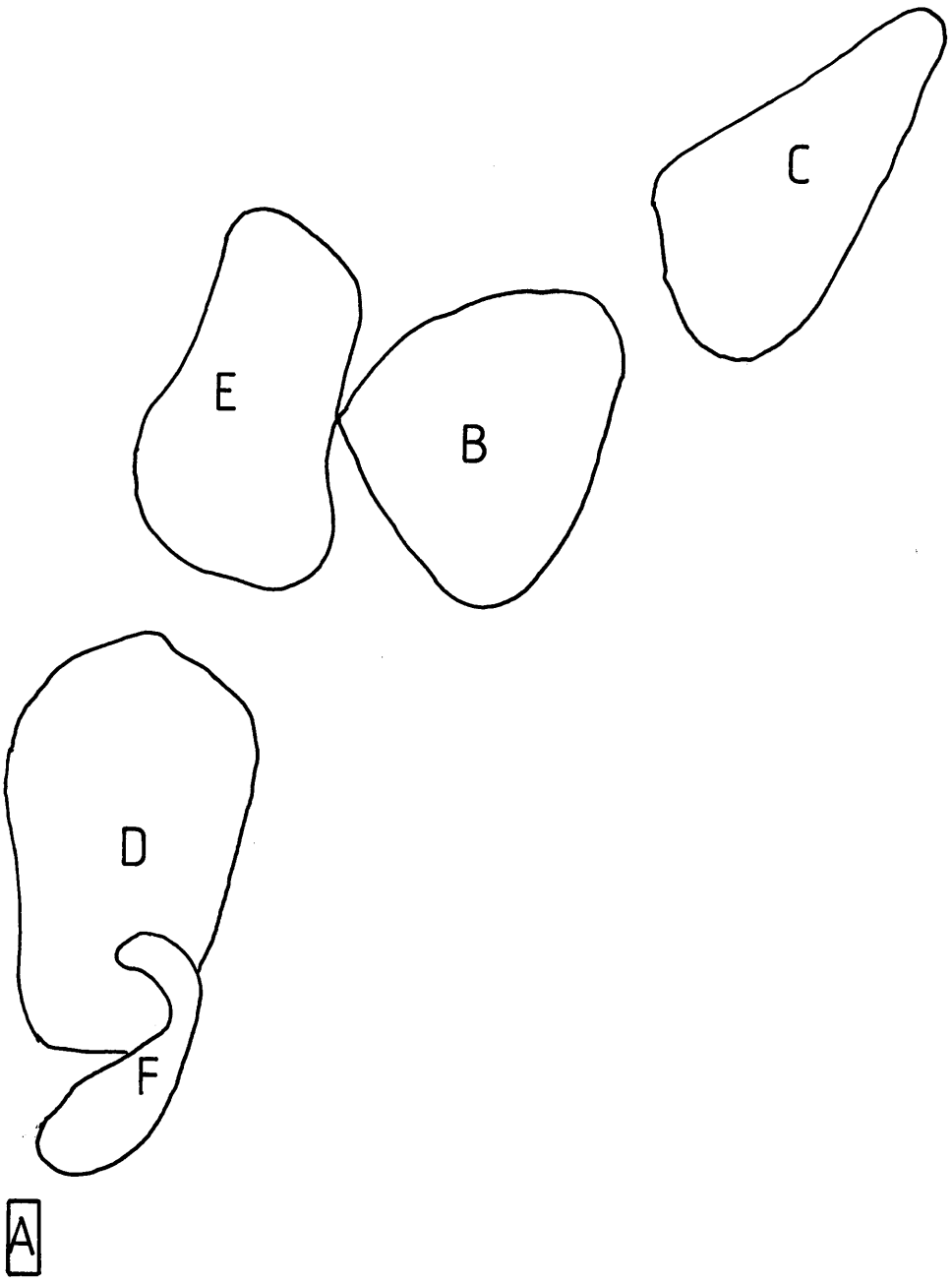
Overlay: indicates general groupings of data.

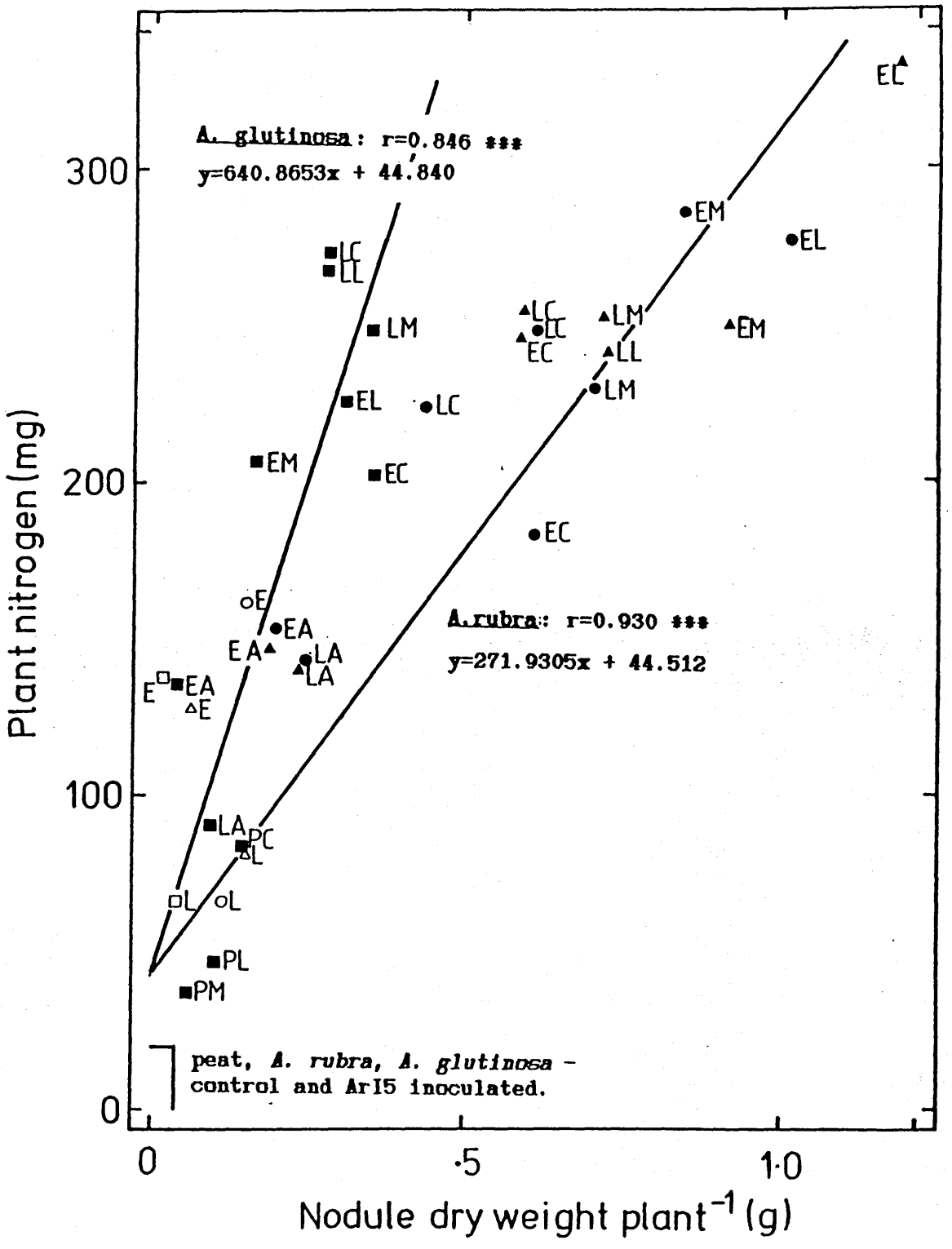
Groups as in Figure 22 except-

A: Peat, *A. rubra* and *A. glutinosa* uninoculated and inoculated with ArI5.

F: Peat, *A. glutinosa* except those included in group A.

Overlay Figure 23




A. glutinosa: r=0.846 ***
y=640.8653x + 44.840
A. rubra: r=0.930 ***
y=271.9305x + 44.512
peat, A. rubra, A. glutinosa -
control and ArI5 inoculated.
Plant nitrogen (mg)
Nodule dry weight plant^{-1} (g)
0
100
200
300
0
·5
1·0

A. glutinosa where differences in growth were more due to differences in nodule specific activity.

In summary, all the factors examined influenced plant growth but to different extents. Soil type had the largest effect followed by inoculation and source of *Frankia* inoculum. Plant genotype generally had the smallest effect on dry weight and nitrogen accretion and interaction between all 3 factors was also evident.

3.3.3 Effect of soil extracts on *Frankia* growth *in vitro*

The above results demonstrate clearly that soil factors can affect nodulation and subsequent fixation and that peats appear to exert a particularly powerful adverse effect. The possibility that substance(s) in the different soils may affect growth of *Frankia* directly was examined by comparison of the effects of aqueous extracts from peats and a mineral soil (Table 44) on the growth of *Frankia in vitro*.

Two strains were grown in BuCT medium to which extracts of soils of the above soil types were added: 1.2.23[Q](b) isolated from Wauchope, a peat site where nodulation was not greatly impaired and 1.2.19[Q] isolated from South Yorks 9, a mineral soil site supporting good nodulation. These two strains were chosen so that any adaptations to either the peat or mineral soil types could be identified.

The results of a preliminary experiment to establish the growth curves for these two strains when grown in BuCT medium are shown in Figures 24 and 25. These curves permitted selection of an appropriate harvest time for the soil extract - *Frankia* growth experiments. The most appropriate point for harvest is about the middle of the logarithmic growth phase so that differences between controls and treatments would not be lost as the growth rate slowed at the stationary and decline phases. 1.2.23[Q](b) showed clear lag, logarithmic, and decline phases with a short stationary phase. Culture pH rose rapidly during the logarithmic phase to reach a maximum close to the stationary phase and then fell during the decline phase. The growth of 1.2.19[Q] was much slower with a short (if any) lag phase and a logarithmic phase; medium pH rose throughout the growth period.

Table 45 shows the *Frankia* protein yield of flasks, containing BuCT and the appropriate extract, previously inoculated with 1.2.23[Q](b) (a strain from a peat site) or 1.2.19[Q] (a strain from a mineral soil site) and harvested after 25 days growth. t tests of the means show that inclusion of an extract from Loch Ard peat had no significant ($p < 0.050$) effect on the growth of 1.2.23[Q](b). The inclusion of extract from Wauchope peat, however clearly stimulated growth to approximately nine times that in BuCT alone, as did the inclusion of a soil extract from Wolfson Hall; there was no significant difference between the two means. t tests of means show that the inclusion of an extract of peat from Wauchope had no significant effect on growth of 1.2.19[Q] but the inclusion of an extract of soil from Wolfson Hall clearly stimulated growth, although not to as great an extent as in 1.2.23[Q](b). The inclusion of an

Table 44: Characteristics of soils from which extracts were prepared.

Site	Location	Soil Description	Dominant Vegetation
Wolfson Hall	NS555695	Agricultural loam	*A. rubra* and grasses
Loch Ard	NS511922	Peat	*Myrica gale*, *Calluna vulgaris* and *Mollinia spp.*
Wauchope	[1]	Deep Peat	Mollinia spp. approx 750 metres from an *A. rubra* plantation

[1] See Table 1 for details.

Table 45: Effects of soil extracts on growth of *Frankia* in BuCT medium.

Strain and source of extract	Protein per flask (41ml) µg ± S.E.[1]	pH of culture medium
1.2.23[Q](b)[2]		
Control (no extract)	94.6 ± 18.34	7.0
Loch Ard (peat)	73.5 ± 7.74	6.8
Wauchope (peat)	862.9 ± 16.45	7.7
Wolfson Hall (loam)	935.1 ± 22.36	7.7
1.2.19[Q][3]		
Control (no extract)	46.5 ± 5.73	6.8
Loch Ard (peat)	7.9 ± 1.04	6.8
Wauchope (peat)	41.4 ± 5.16	6.9
Wolfson Hall (loam)	217.8 ± 12.06	7.1

[1] Figures are means of duplicate measurements on the contents of 11 replicate flasks.
[2] Isolated from *A.rubra* growing on a peat at Wauchope (see Tables 1 for site details).
[3] Isolated from *A.rubra* growing on a mineral soil at South Yorks. 9 (see Table 1 for site details).

Figure 24: Growth of *Frankia* strain 1.2.23[Q](b) in BuCT.

Cultures were grown in 40mls medium in 100mls flasks under non-shaking conditions and at a temperature of 28°C for 25 days. *Frankia* protein at harvest ●——● , medium pH △- - -△ .

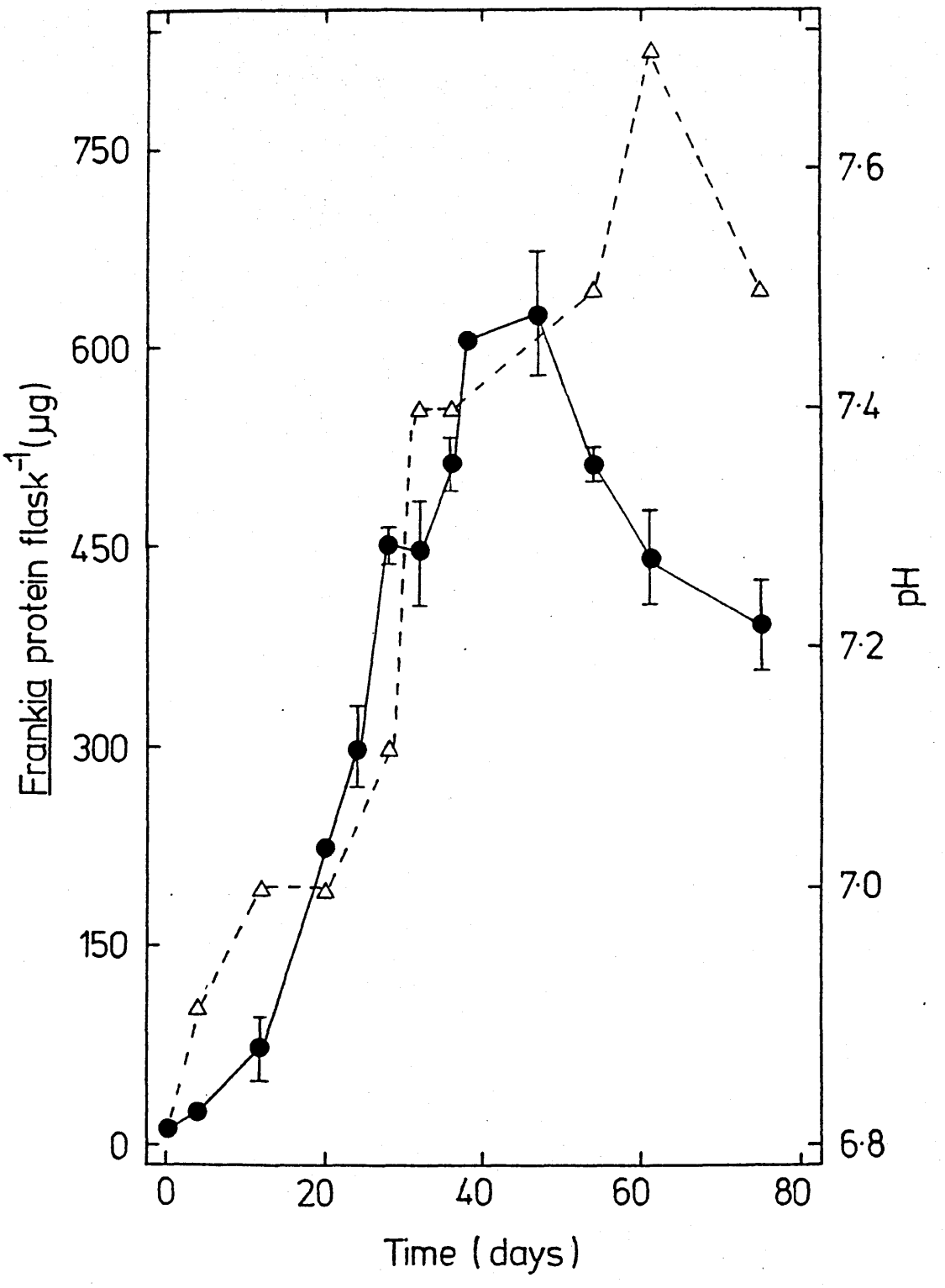
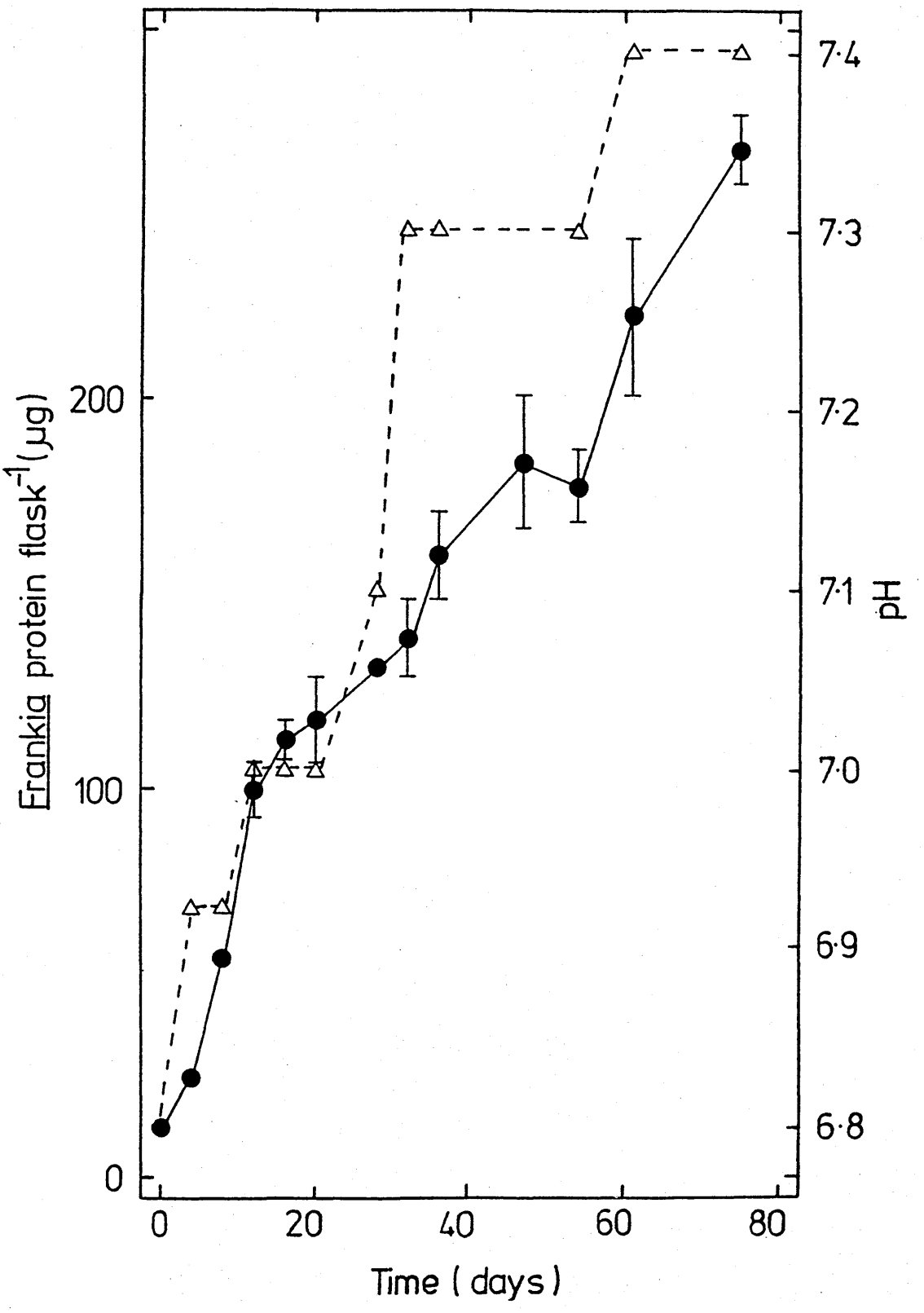

750
600
450
300
150
0
Frankia protein flask^{-1}(µg)
7·6
7·4
7·2
7·0
6·8
pH
0
20
40
60
80
Time (days)

Figure 25: Growth of *Frankia* strain 1.2.19[Q] in BuCT.

Cultures were grown in 40mls medium in 100mls flasks under non-shaking conditions and at a temperature of 28°C for 25 days.


Frankia protein flask^{-1}(µg)
200
100
0
0
20
40
60
80
Time (days)
7·4
7·3
7·2
7·1
7·0
6·9
6·8
pH

extract from Loch Ard peat, however, clearly inhibited the growth of 1.2.19[Q] and the protein yield from each flask was less than the original inoculum.

With both strains, therefore, the inclusion of an aqueous extract from a mineral soil at Wolfson Hall greatly stimulated growth. The inclusion of an aqueous extract from a peat at Loch Ard had no effect on the growth of 1.2.23[Q](b), the strain isolated from a peat site but inhibited completely the growth of 1.2.19[Q], the strain isolated from a mineral soil site. The inclusion of an aqueous extract from a peat from Wauchope stimulated considerably the growth of 1.2.23[Q](b) but had no effect on the growth of 1.2.19[Q]. There are indications from these results, therefore, that firstly some mineral soils contain water soluble substances which stimulate the growth of *Frankia* and secondly that some peats contain water soluble substances which can either stimulate, have no effect or inhibit the growth of *Frankia* depending upon the strain.

Attempts to grow 1.2.23[Q](b) in the different extracts without BuCT medium were unsuccessful. The addition of sodium propionate alone, a carbon source which this strain had been demonstrated to utilise previously (Tables 13 to 15), had no effect.

3.3.4 Assessment of competition between sp^- and sp^+ Frankia for nodulation of *A. rubra*

In addition to soil factors there are a number of biotic factors which may influence endophyte survival and infection. Of particular interest to field applications of *Frankia* are the effects of competition between indigenous and introduced strains for nodulation of host plants. In this experiment, attempts were made to assess strain competition for nodulation using spore formation as a morphological marker.

A. rubra was grown in nitrogen free Perlite culture after inoculation with either ArI5 (sp^+) or a crushed nodule (sp^+) preparation. After they had reached a suitable size, plants were selected for outplanting into soil known to give rise to sp^+ nodules and a number harvested both to assess the relative effectivity of the strains for nitrogen fixation and to confirm the spore identity of nodules. All nodules examined from plants inoculated with ArI5 were sp^- and from plants inoculated with a crushed nodule preparation sp^+.

The results of the growth assessments of plants prior to planting in soil are shown in Table 46. There were significant ($p < 0.001$) effects of inoculum on plant dry weight and plant nitrogen with ArI5 inoculated plants having a plant dry weight and plant nitrogen content approximately 4 times that of crushed nodule inoculated plants. Plant nodule dry weight, however, was similar in plants inoculated with either inoculum. Differences in plant dry weight and plant nitrogen were due to the significantly greater nodule specific activity of ArI5

(sp^-) inoculated plants as measured by ratios of plant / nodule dry weight and plant nitrogen / nodule dry weight ($p < 0.001$) compared to those inoculated with crushed nodules (sp^+). Nodule numbers were significantly greater ($p < 0.001$) on plants inoculated with crushed nodules.

Plants from each inoculated group were transplanted into separate plots containing a soil from Lennox Forest which had been collected from close to the site of harvest of the crushed nodules used in this experiment and which was known, therefore, to give rise to predominantly sp^+ nodules. Plots containing plants originally inoculated with ArI5 were watered with a dilute solution of the inoculum.

The percentage of nodules containing sp^+ lobes in post inoculation infections of plants is shown in Table 47. A number of individual plants in both ArI5 (sp^-) inoculated and uninoculated plots were identified which had nodules which were sp^+ and nodules which were sp^- ; nodule lobes within the same nodule, however, were always of the same spore type. The existence of both types of nodule on plants growing in the uninoculated plots makes it likely that the soil used in this experiment contained *Frankia(e)* capable of causing the development of both types of nodule. Inoculation with ArI5, however, reduced the incidence of sp^+ nodules considerably with only a single nodule being identified as being sp^+. This indicates that inoculation of plants and plots with a selected strain did in this case lead to the formation of a greater proportion of new nodules of similar character to the introduced strain. It appears, therefore, that the

Table 46: Growth of *A. rubra* inoculated with sp^+ or sp^- inoculum.

	Inoculum		
	ArI5(sp^-)	Crushed nodules(sp^+)	P[1]
Plant d.w. (g)	8.5 ± 0.74	2.0 ± 0.23	***
Nodule d.w.	0.110 ± 0.0086	0.094 ± 0.0011	NS
Nodule no.	15 ± 1.1	64 ± 11.0	**
Plant / nodule d.w.	80.4 ± 7.88	21.3 ± 0.91	***
N(mg)g^{-1} plant d.w.	14.1 ± 0.71	13.6 ± 0.68	NS
Plant N (mg)	117.5 ± 6.23	27.3 ± 3.55	***
Plant N / nodule d.w.	1.11 ± 0.102	0.29 ± 0.014	***

Figures are means of 8 replicates, All plants harvested were nodulated,
P[1] indicates probability at which means are statistically significant from each other using t-tests,

Table 47: Effects of inoculation with a sp^- *Frankia* strain on spore production in post-inoculation infections.

	Percentage nodule clusters with sp^+ lobes	
Plot no.and (plant no.)	Inoculated	Non-inoculated
1 (1)	20	20
1 (2)	0	0
1 (3)	0	60
2 (1)	0	0
2 (2)	0	20
2 (3)	0	0
Mean	3.3	16.7

Data are for 5 nodules from each of 3 plants in each plot, Nodule clusters selected for examination were harvested from the lower half of the root system; away from nodules which may have developed prior to outplanting, Two lobes were examined from each; in all cases the spore nature of lobes from the same nodule cluster were identical,

introduced strain largely out-competed the indigenous strains already present in the soil for nodulation of *A. rubra*.

3.3.5 Nursery inoculation of *A. rubra* with selected *Frankia* strains

Previous experiments in nitrogen-free culture in growth cabinets (3.2.2, 3.2.3, 3.2.4) clearly showed that inoculation of *Alnus* with different *Frankia* strains could result in significantly different nodulation, growth and nitrogen fixation. This experiment was designed to show whether inoculation of *A. rubra* in the nursery would lead to better growth and whether differences which existed in growth room experiments were maintained under field conditions. Strains which had been shown in growth cabinet tests to be highly effective in nitrogen fixation were used to inoculate *A. rubra* and the growth of inoculated plants was compared with plants inoculated with a crushed nodule preparation and with uninoculated controls. The conditions of this experiment approximated, as closely as possible, to normal commercial practice.

The data in Tables 48 and 49 show that inoculation clearly affected plant growth and inoculated plants had a significantly ($p < 0.001$) higher plant dry weight and higher plant nitrogen with up to approximately 3 times the dry weight and 9 times the nitrogen content of uninoculated controls. Particularly interesting were the considerable differences in the nitrogen content per gram dry weight of inoculated plants - approximately twice that of uninoculated plants. At harvest, some of the control plants showed a low level of

Table 48: Growth of inoculated and uninoculated *A.rubra* in a nursery.

Treatment	Plant dry weight(g)	Ratio root to shoot d.w.	Nodule dry weight(g)	No. of nodules	Nodule disribution[1]
NPK	1.0 a	0.38 a	0.007 a	1 a	p
PK	1.0 a	0.39 a	0.016 a	2 a	p
Crushed nodules	2.7 b	0.25 b	0.179 b	69 b	3
ArI4	2.8 b	0.27 b	0.107 c	21 c	2
1.2.5[Q](b)	3.2 b	0.34 a	0.189 b	11 d	2
S.E.	0.20	0.022	0.0147	2.9	

Figures are means of 20 replicates. All inoculated plants examined were nodulated. Means followed by the same letter are not significantly different ($p < 0.050$) from one another using Duncan's Multiple Range Test. Where means are not followed by a letter Analysis of Variance showed no significant effect ($p < 0.050$) of strain on the parameter. S.E. indicates standard error of the treatment mean. Analysis of variance table is shown in Table 48S, Appendix 4.

[1] Nodule distribution is coded as follows: p, confined to periphery of root system; 2, confined to upper 30% of root system; 3, extends throughout upper 60 to 70% of root system.

Table 49: Nitrogen content of inoculated and uninoculated *A. rubra* grown in a nursery.

Treatment[1]	N(mg)g plant d.w.[2]	Plant N(mg)[3]
NPK	12.89	12.89
PK	9.62	9.62
Crushed nodules	20.59	55.59
ArI4	21.72	60.82
1.2.5[Q](b)	22.47	71.90

[1] N, P, and K indicate application of nitrogen, phosphorus and potassium respectively at planting.
[2] Figures are means of duplicate determinations on each of 3 separate batches.
[3] Calculated by multiplying the mean plant dry weight for each treatment by the mean nitrogen content per gram dry weight.

nodulation although infections were always on the periphery of the root system. Observations made at harvest suggest that these infections were due to the spread of endophyte from inoculated plots, or from adjacent, unsterilised soil.

With the exception of plants inoculated with 1.2.5[Q](b) inoculation had a significant ($p < 0.001$) effect on root to shoot ratios, reducing them considerably.

The distribution of nodules formed as a result of inoculation with 1.2.5[Q](b) was, as in Perlite in batch 1 (Table 19), limited to the upper 30% of the root system. In contrast the distribution of nodules formed by inoculation with either crushed nodules or ArI4 was different to that observed in Perlite. Previously, in Perlite, nodules formed by inoculation with ArI4 were limited to the root crown whereas in the nursery they were distributed over the upper 30% of the root system. Even larger differences occurred in the distribution of nodules formed by inoculation with crushed nodules. Previously observed in Perlite to be limited to the root crown, in the nursery they occupied the upper 60 to 70% of the root system. Differences in the number of nodules formed was also evident. Similar numbers were observed in 1.2.5[Q](b) inoculated plants to those grown in Perlite but nursery grown ArI4 inoculated plants had approximately 3 times the number of those grown in Perlite although plant dry weight values were similar at harvest. In crushed nodule inoculated plants, however, even larger differences were observed with over 20 times the number of nodules formed in the nursery than previously observed in Perlite despite the plants only being about twice as large at harvest.

Table 50: Growth of *A. rubra* in the nursey and growth cabinets after inoculation with *Frankia*.

Inoculum	Plant N / nodule d.w. (% of ArI4)	
	Nursery[1]	Growth room
ArI4	0.479	1.08
1.2.5[Q](b)	0.330 (68.9)	0.76 (70.4)
Crushed nodules	0.257 (53.7)	0.72 (66.7)

1 Nitrogen content of PK control plants subtracted from nursery-grown plants so plant N / nodule d.w. is an estimate nodule specific activity.

In addition to these effects of inoculation there were a number of differences between plants inoculated with the different strains. Although plant dry weight and plant nitrogen was similar in all inoculated plants, those inoculated with ArI4 had significantly ($p < 0.050$) lower nodule dry weights than those inoculated with either 1.2.5[Q](b) or crushed nodules, suggesting that the specific activity of nodules formed by this strain was superior. This conforms to observations made previously when inoculated plants were grown in Perlite within growth cabinets (Table 22). Comparisons of nodule specific activity estimated as the ratio plant nitrogen / nodule dry weight (Table 50) show that although the amount of nitrogen fixed per unit nodule weight in the nursery was less than in the growth cabinets the relative order of specific activity of nodules formed by inoculation with the different strains remained the same and that differences between values were similar when expressed as a percentage of ArI4.

4.0.0 Discussion

4.1.0 Isolation of *Frankia*

Detailed comparisons of the effectivity of different techniques for the isolation of *Frankia* have not been reported previously. The comparison of 3 isolation techniques in this study, utilising a variety of isolation media, showed that both isolation technique and medium could influence the success of isolation attempts depending on the source of nodules (Tables 3 to 5). The differential filtration technique was the most successful. Isolates were not obtained using other techniques when this technique was unsuccessful. In some cases specific medium requirements for the isolation of the endophyte were demonstrated and these requirements were also shown to be influenced by the choice of isolation technique. Using the differential filtration technique to isolate *Frankia* from Diemen, for example, the addition of propionate to the medium was stimulatory whilst its addition to medium in conjunction with the sucrose density fractionation technique had little, if any, effect (Table 3). The observation that a total lipid fraction was necessary for the isolation of *Frankia* from sp^- nodules collected from the field at Diemen and from nodules of water-culture plants inoculated with crushed nodule preparations from the same source confirms earlier reports of specific medium requirements for growth in culture of the endophyte from this site (Quispel et al. 1983).

A notable feature of all isolation attempts was the difficulty experienced in isolating *Frankia* from field collected sp^+ nodules compared with sp^- nodules. This is discussed in greater detail below

(p. 204). However, in these first experiments to compare isolation techniques it did prove possible to isolate the endophyte from sp^+ nodules from one of the field sites, Balmaha, from nodules of water-culture grown plants inoculated previously with crushed preparations of Balmaha *A. glutinosa* nodules (Table 5). However, this isolate could not be maintained in liquid culture (A.J.P. Burggraaf, personal communication). The reasons for this partial success are unclear. Other isolation attempts were made from field-collected nodules from Balmaha stored at -20°C prior to processing for *Frankia* isolation. It is conceivable that freezing may have affected the sp^+ nodule *Frankia*(e) more than the sp^- nodule *Frankia*(e), making the former non-viable. However, it is probable that several *Frankia* strains occur at the Balmaha site. The nodules produced on water culture plants following inoculation with crushed nodules may have contained different strain(s) from the field nodules used in isolation attempts; or the strains which proliferated in the nodules of water culture plants may have been different from strains which predominate in the nodules in the field. The observation that the addition of propionate to the medium was stimulatory for the isolation of *Frankia* from field nodules whilst the same addition was inhibitory for *Frankia* isolation from water-culture nodules (Table 5) suggests that a different endophyte may have been present. There are several reports of the occurrence of different strains at the same site (eg. Normand and Lalonde, 1982; Lechevalier *et al.*, 1983; Hafeez *et al.*,1984; Lechevalier and Ruan, 1984) and there is even a report of different strains occurring within a single nodule (Benson and Hanna, 1983). The results obtained here provide further evidence for multiple strains of *Frankia* within nodules from a single plant.

In conclusion, it is clear that different *Frankia* strains, with different demands for growth may be isolated using different techniques and media. Furthermore, it is probable that the isolation of endophytes in different areas may be favoured by specific medium / technique combinations.

The general applicability of the differential filtration technique for *Frankia* isolation was indicated further by the successful isolation of a number of *Frankia* isolates from *Alnus* and *Hippophae* from different field sites in Britain, North America and Sweden (Table 7). Although isolates were obtained using a number of different media the most successful medium was Bu which contains propionate. Notable among the media used for the successful isolation of *Frankia* was a medium originally developed by Pommer (1959). There are no other reports of this medium being used successfully to isolate *Frankia* and this observation provides further confirmation of the early success of Pommer in isolating *Frankia* from *Alnus*. The isolation medium preferences for certain strains was confirmed further (Table 8). Such variation in the requirements of particular strains for specific nutritional components are well established for *Frankia* growth *in vitro* and are discussed later (in 4.2.0).

Irrespective of isolation medium, in most cases growth of isolates could be obtained in liquid culture on a standard cultivation medium, BuCT. However, some failed to grow (Table 11) and also did not show growth when transferred back into medium of the same composition used for isolation. Possible explanations are that either initial growth requirements are different from those which support growth later or, more likely, that limited growth on agar plates is supported initially

by small quantities of a substance(s) essential for growth which are present in nodule fragments in the agar.

Growth of *Frankia* colonies on isolation plates could be observed in the same period (2 to 5 weeks in most cases) as reported previously (eg. Berry and Torrey, 1979; Normand and Lalonde, 1982; Zhang *et al.*,1984). The observed pigmentation (Table 11) of some isolates has been widely reported (eg. Baker, 1982) although a distinction between colony pigmentation and excretion of water-soluble pigments from the colonies is seldom made.

Three distinct colony forms were recognised among isolates (Table 11). Detailed studies of the relationship between *Frankia* genotype and colony form have not been reported but variations depending on growth rate have been observed. For example, Baker and Torrey (1980) observed both diffuse and compact colony forms in AvCI1 and noted that the form was related to the medium, with media supporting better growth usually resulting in a more compact colony. Burggraaf and Valstar (1984) also observed different colony forms and described both compact and more diffuse forms. However, they made no comment on a possible relationship between form and growth. In the present study the colony forms were undoubtedly medium and growth rate dependant (Table 11). Such plasticity of colony morphology clearly renders this characteristic unsuitable for *Frankia* strain classification or identification.

Previous reports of hyphal diameters in *Frankia* mainly range from 0.5 to 1.5 μm (eg Callaham *et al.*, 1978; Lechevalier *et al.*, 1983). The observation in the present study of isolates with larger hyphal

diameters, up to 2.8 μm (Table 9), is therefore of interest. However, such detailed characteristics of isolates often go unreported. Furthermore, those reports which have been made are usually of *Frankia* in culture not on isolation plates, and it is conceivable that hyphal dimensions in agar could be different to those in liquid culture.

The differences frequently observed in sporangial production (both in numbers and size), by colonies on isolation plates, could be due to genotypic variation between the colonies or to phenotypic variations resulting from local differences in the medium on the isolation plate. Given the numerous reports of media effects on sporulation (eg Baker and Torrey, 1980; Burggraaf, 1984), the latter possibility seems likely, particularly as it is difficult to distinguish between the morphology of such colonies after sub-culture.

4.2.0 Characteristics of strains

Why strains obtained from sites which contained sp^+ nodules and from sites which contained sp^- nodules only gave rise to sp^- nodules following inoculation of test plants is not understood. Most investigators likewise have been unsuccessful in isolating *Frankia* from sp^+ nodules. The only two reports of successful isolations from sp^+ nodules are those of Normand and Lalonde (1982) and Burggraaf (1984). Normand and Lalonde (1982) reported the isolation of strains from sp^+ nodules of *A. rugosa* and *A. crispa* which showed *in vivo* spore production on re-inoculation of the host plant. These 'sp^+' strains are claimed to produce only low numbers of sporangia *in vivo*

and these became fewer the longer the strains were maintained in culture (P. Normand, personal communication). Burggraaf (1984) reported the isolation of a number of strains from both sp^+ and sp^- nodules of *A. glutinosa*. However, on re-inoculation onto host plants he observed that on several occasions strains which had been isolated from sp^- nodules caused the development of nodules which were sp^+. In contrast, he observed only a few sporangia in nodules formed as a result of inoculation of test plants with strains isolated from sp^+ nodules (A. J. P. Burggraaf, personal communication).

The failure in this study to isolate *Frankia* from sp^+ nodules which caused the development of sp^+ nodules on re-inoculation onto host plants could be due to a number of possibilities. Firstly, although sp^+ nodule lobes were identified at the sites from which nodules were collected they may not have been included in the isolation material. However, this is unlikely as lobes for microscopic examination were always selected from the same nodules as the lobes used for isolation. Secondly, it is possible that both sp^+ and sp^- strains existed within the nodules and sp^- strains were selected for during the isolation process - evidence for the presence of different strains within a single nodule lobe has been presented previously (Benson and Hanna, 1983). Thirdly, the process of isolation from the nodule may have changed the *Frankia* genome so that the ability to form sporangia *in vivo* was lost. The possibility of such spontaneous mutation was suggested by Burggraaf (1984) to explain the plasticity of spore production *in vivo* by isolates as discussed above. In *Streptomyces*, sporulation is variable and its absence can be brought about by plasmid loss (Ensign, 1978). Plasmids are known to occur in *Frankia* (Normand *et al.*, 1983) and it is possible that plasmid loss could be

involved in the loss of the ability to sporulate '*in vivo*'. However, the retention of the ability of all strains to sporulate '*in vitro*' perhaps makes such an explanation less likely. Finally, and contrary to previous proposals that the sporing nature of a strain is genetically determined (Dijk, 1978; Normand and Lalonde, 1982; VandenBosch and Torrey, 1985) there is the possibility that spore formation '*in vivo*' is influenced by unknown factors within the nodule (Torrey, 1987). If this hypothesis were correct it would go some way to explaining the observations of P. Normand and A. J. P. Burggraaf discussed above - as differences in test plant growth conditions would influence sporangium formation. The main evidence for such a hypothesis comes from studies *in vitro*. Firstly, all strains that have been isolated show the ability to form sporangia under the correct conditions. Secondly, it has been shown that this expression may be influenced by the addition to the growth medium of specific compounds (eg Lalonde and Calvert, 1979; Perradin *et al.*, 1983; Tisa *et al.*, 1983). Such a hypothesis could also explain the reported spatial distribution of different spore types (Dijk, 1984), local environmental conditions influencing plant growth and thus conditions within the nodule. However, the results obtained in this study tend to support the 'genetic' rather than the 'environmental' hypothesis. Plants inoculated with sp^+ crushed nodules gave rise to sp^+ nodules in growth conditions identical to those in which plants inoculated with sp^- crushed nodules gave rise to sp^- nodules (Table 16). Furthermore, the addition of an extract from sp^+ nodules to sp^- inoculum did not induce sporangial production (Table 23).

The ability to utilise different carbon sources for growth permitted a preliminary characterisation of *Frankia* strains (Tables 13 to 15).

The utilisation of propionate, acetate and Tween by many strains, indicates the existence of a pathway for the metabolism of fatty acids and their derivatives, and is in agreement with previous reports for other *Frankia* strains (see introduction). The failure of a number of strains to use all 3 of these carbon sources is in agreement with observations by Benson and Hanna (1983) and indicates either that the metabolic pathways differ in different strains or that transport systems for some metabolites are not always active. Succinate utilisation by some strains in this study suggests that TCA cycle enzymes are present and the utilisation of glucose suggests that enzymes of glycolysis are also present. Previous reports of the utilisation of the TCA intermediates, including succinate (eg Benson and Hanna, 1983; Burggraaf and Shipton, 1983) are less common than those demonstrating fatty acid metabolism and reports of glucose utilisation are also uncommon (eg Shipton and Burggraaf, 1983; Burggraaf, 1984; Lechevalier and Ruan, 1984). However, a recent report by Lopez and Torrey (1985) showed the presence of the enzymes of glycolysis in both vesicle cluster preparations and pure cultures of *Frankia* and the inability of many strains to utilise carbohydrates has been suggested by Lechevalier and Ruan (1984) to be due to the lack of a suitable transport system. Such suggestions are supported by observations, confirmed in the present study, that Tween can act not only as a carbon source but also to facilitate uptake of other carbon compounds (Lechevalier and Ruan, 1984), presumably by increasing the permeability of cell membranes (Tjepkema *et al.*, 1986).

A surprising number of strains failed to utilise any of the carbon sources tested. These strains were all able to grow satisfactorily in BuCT, however, and it seems likely that they either used casamino

acids as a carbon source or that there is some synergism between the different compounds present; as has been reported, for example, for Tween and glucose (Lechevalier and Ruan, 1984).

A particularly interesting result was the ability of strains ArI3 and ArI5 to grow on a carbon-free medium. Such growth could result from the utilisation of carbon reserves in the mycelium, or conceivably might be due to CO_2 fixation supported by oxidation of H_2 evolved by nitrogenase - most cultures examined produced some vesicles, although their activity in nitrogen fixation was not determined. The only previous demonstrations of CO_2 fixation by *Frankia* were in CpI1 by Shipton and Burggraaf (1983) under enriched CO_2 and H_2 atmospheres, but with low pO_2. Akkermans *et al.* (1983) demonstrated increased growth by AvCI1 when CO_2 was present in an aerobic atmosphere; such stimulation of growth by enhanced levels of CO_2 is a common feature of microbial metabolism.

Two broad factors determine how much nitrogen will be fixed by a particular *Frankia* strain - host plant combination (ie the effectivity of the symbiosis). These factors are a) nodule growth per plant or nodule productivity and b) nodule specific activity - nitrogen fixed per unit weight of nodules (Reddell and Bowen, 1985). Most previous studies of the effectivity of *Frankia* symbioses have not attempted to separate different effects of the *Frankia* strain on the two factors.

Reports by Normand and Lalonde (1982) and Simonet *et al.* (1985) concluded that cultured strains, isolated from sp^+ nodules were less effective in symbiosis than sp^- strains, confirming earlier reports by

Hall *et al.* (1979) and Maynard (1980) who used crushed nodules as inocula. These conclusions, however, were based solely on measurements of either shoot height, total biomass or nitrogen content and no differences between the strains were ascribed to the two main determinants of strain effectivity. However, Dijk and Merkus (1976) did measure specific activity and found no difference in the specific activity of nodules formed after inoculation of *A. glutinosa* with either sp^+ or sp^- nodules. That sporangial development can result in reduced nodule specific activity was, however, convincingly demonstrated by VandenBosch and Torrey (1984) who showed also that in nodules of *Comptonia peregrina* and *Myrica gale* this was associated with an increased respiratory cost for nitrogen fixation.

As it did not prove possible in the present study to isolate strains which formed sp^+ nodules, growth comparisons were made of plants with sp^+ or sp^- nodules formed following inoculation with sp^+ crushed nodules or cultures of sp^- strain. In two experiments (Tables 16, 17 and 46) the specific activity of nitrogen fixation of sp^+ nodules from two sources was shown to be much lower than of sp^- nodules, thus confirming most previous observations. However, in one experiment (Table 16) despite the lower specific activity of sp^+ nodules, overall nodule effectivity - total nitrogen fixed per plant - was similar to the sp^- nodules due to differences in nodule productivity per plant. These experiments illustrate the different contributions which these determinants can make to overall strain effectivity.

Although many previous reports have failed to identify differences in effectivity between sp^- strains (see Introduction), in this study large differences were observed (Tables 20 and 21). Importantly,

North American *Frankia* strains were no more and in some cases less effective than British strains. The most effective associations were homologous and, in general, the result of a high weight of nodules per plant rather than a high specific activity. These experiments, therefore, confirm further the importance of measuring separately the effects of *Frankia* strain on nodule productivity and on the specific activity of nitrogen fixation. Metabolic events that were not assayed such as a high respiratory rate or more efficient coupling between nitrogenase and reductant supply, could explain higher rates of nitrogen fixation by some strains. It is more difficult, however, to understand why strains differed in their ability to stimulate and sustain enhanced nodule growth, particularly since strains that stimulated best nodule growth were not usually those producing nodules with the highest specific activity of nitrogen fixation. A possible explanation is that it reflects a balance in the nodule between the demand for assimilates by *Frankia* to support nitrogenase activity and by the host cells for ammonia assimilation on the one hand, and on the other hand the demands of the nodule cells for maintenance, expansion and for meristematic activity. Too high a demand for assimilates for the nitrogen fixation process, as would occur in nodules of high specific activity, could limit the availability of assimilates for nodule growth. Another explanation is that some strains produce or stimulate the production by the host plant of additional quantities of growth substances *in vivo*, which result in nodule growth. A small production of indole-3-acetic acid by cultures of AvcI1 has been reported (Wheeler *et. al.* 1984). Although nodule productivity is the most important determinant of strain effectivity in homologous associations nodule specific activity is the most important determinant in heterologous associations (Figures 8 and 9). It

appears that with the strains examined, the limitation on symbiotic nitrogen fixation and hence growth in these heterologous associations is due, at least in part, to restriction of nodule growth. It would appear that some factor(s), which in the nodules of homologous associations permit or promote nodule growth broadly in line with the intrinsic ability of a strain well adapted to that species to fix nitrogen in symbiosis is missing from the nodules of heterologous associations. The observation that one strain 1.1.8[Bu] was able to form both homologous and heterologous assocations with only a small reduction in effectivity is of importance. Such a strain could be particularly suitable for use as a field inoculum in situations where more than one *Alnus* species is grown.

Effects on other parameters were also observed. There are no previous reports of strain effects on root to shoot ratios. However, in the present study differences due to *Frankia* strain were demonstrated (Tables 20, 23and 25). They were, however, infrequent and were not large. Furthermore, they were not constant. For example, the significant differences in the root to shoot ratios of plants in Batch 5 (Table 25) were not encountered in the previous comparison of these strains (Table 20). However, differences in root to shoot ratios may often be ascribed to the growth stages of plants (Evans, 1972). This seems a likely explanation here because of the substantial differences in the growth of plants in batches 2 and 5. Such variability suggests that it would be premature from these experiments to draw definite conclusions concerning the effects of *Frankia* strain on the root to shoot ratio.

It is clear from the growth experiments that the strains isolated from *A. rubra* generally were better adapted to symbiosis with *A. rubra* than with *A. glutinosa*, and vice versa. As *A. rubra* is an exotic species and was brought to Britain as fruits the question arises as to how the adapted strains could be present in all the localities from which strains were isolated. It is possible that either a particular strain(s) could be selected in the field by a particular host or that a gradual alteration in the strain genotype occurs during symbiosis. Although there are no reports in the literature to support the latter possibility Burggraaf (1984) reported evidence which he suggests indicates that selection of specific strain(s) occurs during infection. Although he initially categorised *Frankia* strain LDAgp1 as having only a marginal nodulation capacity, reisolates which he obtained from the occasional nodules formed on alder plants inoculated with this strain showed normal nodulation 2-3 weeks after inoculation onto test plants. It is presumed that similar strain selections and/or modification must have taken place to suit indigenous *Frankia* strains to *A. rubra* at the different study sites. However, evidence apparently contradicting this was obtained from the study of different *Frankia* strains, isolated from *A. rubra* and *A. glutinosa* growing side by side at Rumster 9 (Table 7). The effectivity and nodule specific activity of the strain isolated from *A. rubra*, when inoculated back onto *A. rubra*, was shown to be inferior to that of the strain isolated from *A. glutinosa* and furthermore only 88% of plants were nodulated (Tables 19 and 20). This apparently contradicts the hypothesis that *Alnus* are normally nodulated by a strain(s) best adapted to that species. It is possible that *A. rubra* nodules at this site contain more than one *Frankia* strain and that the strain isolated while easy to grow in culture, happened to be of low effectivity. It is also possible that

the strains isolated from the two species are those which predominated in the nurseries in which they were grown prior to outplanting. *Frankia* appears to spread little in peat (see 3.3) and hence cross-infection with *Frankia* strains from the two species may not have occurred.

Although differences in strains isolated from different species at the same site are interesting the observation that strains isolated from the same site and species were also different is perhaps more so and confirm observations made by Benson and Hanna (1983) from a characterisation of their isolates by means of their protein patterns on polyacrylamide gel electrophoresis and carbon source utilisation patterns.. In the present study for example, of strains 1.1.4[F], 1.1.5[F] and 1.1.2[Q] isolated from *A. glutinosa* growing at Milngavie at least one of them 1.1.2[Q] was different from the others in both pigmentation (Table 11) and the carbon sources utilised (Tables 13 to 15). More importantly, however, and not previously reported were the observed differences in infectivity, effectivity and nodule specific activity of 1.1.2[Q] (Tables 19 to 21) to the other two strains. Because 1.1.4[F] and 1.1.5[F] showed no differences in these characteristics, it is conceivable that they are identical or very similar strains, despite being isolated on separate occasions. Further evidence for co-existence of strains in symbiosis was obtained by the isolation of strains 1.2.5[Q](b) and 1.2.5[P] from the same nodule cluster of *A. rubra* growing at Lennox. Again differences between these strains in pigmentation and the carbon sources utilised were observed. Although inoculation of *A. rubra* with each strain led to the formation of nodules with a similar specific activity the nodule productivity of plants inoculated with 1.2.5[Q](b)

was considerably greater - leading to a greater effectivity i.e. nitrogen fixed per plant. Evidence was also obtained which suggested the presence of further strains within these nodules. On the same occassion as the other 2 strains were isolated another *Frankia*, 1.2.5[Q](a), which formed colonies morphologically very different from the other two appeared on isolation plates but this isolate could not be sub-cultured in liquid culture (Table 12). Furthermore, the observation that inoculation of plants with the strain isolated from this sp^+ site led to the development of nodules which were sp^- also suggests that some strain(s) were not isolated - although arguments against this possibility, implicating environmental effects in nodule spore production, were put forward earlier. Environmental influences certainly certainly affected properties such as infection since although 100% of plants inoculated in the greenhouse with Lennox sp^+ crushed nodule inoculum and grown under combined nitrogen free conditions in Perlite were nodulated at harvest (see 3.3.4), only 83% and 28%of plants similarly inoculated in the growth rooms were nodulated (Tables 19 and 22).

Previous reports have also noted occassional difficulties using crushed nodules as inoculum (eg Bond *et al.*, 1956; Gardner, 1958). Quispel (1954) in an attempt to remove what he believed were soluble inhibiting substances filtered a crushed nodule suspension but reported no difference in the nodulating capacity due to filtering. Later experiments by Baker *et al.* (1979), however, demonstrated convincingly that the nodulating capacity of fractions of crushed nodule inocula from *Elaegnus* and *Myrica* separated on sterile Sephadex G-50 was up to 5 times greater than the crude crushed nodule suspension.

In view of these findings, it was thought possible in the present study that substances within the nodule released on crushing may be responsible for the failure of nodulation of some plants inoculated with crushed nodules and for delayed nodulation of inoculated seedlings (Table 19). Further experimentation confirmed that not all plants were nodulated after inoculation with crushed nodules whereas 100% of plants were nodulated when inoculated with a cultured *Frankia* strain isolated from the same site (Tables 22 to 24). However, the addition of crushed nodule inoculum to the strain homogenate immediately prior to inoculation did not reduce the number of plants nodulated with the strain. Indeed, a significant increase in nodule numbers per plant demonstrated that the mixed suspension was more infective than the cultured strain alone. This result shows in this instance that crushed nodule suspensions do not inhibit infection and, therefore, contradict the results of Baker *et al.* (1979). Two explanations may be offered for this apparent contradiction. Firstly, the inhibitory substances present in crushed nodule preparations only inhibit infection to a limited extent and the infection which occurs in their presence depends on the concentration of endophyte in the inoculum. On this basis, the strain homogenate in the present study would contain sufficient endophyte to overcome any inhibition. Secondly, it is possible the isolated strain was less susceptible to inhibition due to an adaptive change which may have taken place during culture *in vitro*.

The results discussed previously for *A. rubra* were all from experiments in which seedlings were grown from fruits collected from Lennox Forest. The question arises, therefore, whether similar studies, carried out with different plant genotypes of other

provenances of this species will change the effectivity of strains for symbiotic nitrogen fixation, through effects on the determinants of effectivity - nodule productivity and nodule specific activity.

Significant effects of provenance upon strain effectivity have been reported previously by Dawson and Sun (1981) and Carpenter *et al.* (1984) and by Simon *et al.* (1985) who also demonstrated significant variation in the effectivity of strains in combination with 2 clones from the same provenance.

In the present study significant effects of provenance on strain effectivity, as measured by plant dry weight were not observed (Table 28). Standard errors in this experiment were large, however, and it is possible that small differences between provenances may have been overlooked. Only in one case was it possible to observe a significant strain / provenance effect on nodule specific activity. It would appear, therefore, that the choice of provenance has a relatively small effect on strain effectivity. The lower nitrogen content of plant material in plants from McNab's provenance (Table 29), however, may indicate a general reduced effectivity in nitrogen fixation of stains in symbiosis with this provenance.

Further evidence of a more pronounced interaction comes from previous experiments with the strains (Table 20). Here, where symbiosis was with *A. rubra* of Lennox provenance, the specific activity of nodules formed after inoculation with ArI4 was significantly greater than of nodules formed after inoculation with either of the other strains used in this experiment. The failure to observe a similar difference in the

provenance comparisons *per se* indicates that some provenances may have a greater effect on the symbiosis than those tested.

A number of effects of provenance on symbioses, other than on nodule effectivity, were observed. Notable was the delay in nodulation time with the ArI4 / McNabs combination (Table 27), particularly since in a previous experiment with Lennox provenance, ArI4 inoculated plants developed nodules 7 days earlier than those inoculated with 1.2.19[Q] or 1.2.23[Q](b) the other 2 strains tested in this comparison (Table 19). The only previous study to examine the effect of host genotype on nodulation time demonstrated significant differences between 2 clones of the same species as well as showing that both host species and strain genotype were important in determining the time taken for nodules to appear (Nesme, 1985). The selection for fast nodulating strain / provenance or clone combinations in the field could confer considerable competitive advantages, the importance of which has already been demonstrated in *Trifolium* spp. (Hely, 1972).

Clearly, however, the most important factor which will determine the suitability of a strain as a field inoculant is its effectivity in symbiotic nitrogen fixation and this can be greatly influenced by nodule specific activity. Variation in nodule Relative Efficiency (R.E.), reflecting as it does diversion of electron flux through nitrogenase from N_2 to H_2 could be a major cause of differences in specific activity with different strains.

For experimental convenience, R.E. measurements were carried out on detached, nodulated root systems and this approach required careful consideration of the effects of detachment on nitrogenase activity and

hydrogen evolution. Both nitrogenase activity (C_2H_2 reduction) and hydrogen evolution were observed to decrease rapidly over the period of the assay (Figure 12). These observations broadly concur with those of Minchin *et al.* (1983) who showed that respiration fell off rapidly at about the same rate as acetylene reduction. Moreover, they showed also that these decreases were linked to the inhibition of ammonia production and thus represented decreases in actual nitrogen fixation. In the present study, therefore, because the nitrogenase activity of samples was assayed after hydrogen evolution R.E. would have been underestimated. This is of no significance for the data obtained since the R.E. of nodules of all symbioses was very high (in excess of 0.98). It is clear, therefore, that differences in the R.E. of nodules do not make significant contributions to the variations in nodule specific activity observed. This is in agreement with previous reports of high R.E. in actinorhizal plants (see Introduction). The only report of a low R.E. is that of Sellstedt *et al.* (1986) for sp^+ nodules from *A. incana* - this symbiosis also resulted in nodules with a low specific activity.

Hydrogen uptake activity of nodules from all *A.rubra* - *Frankia* associations studied was observed to remain relatively constant over long periods although variation between samples was large (Table 31). These observations are in contrast to the observations of Roelofsen and Akkermans (1979) whose data show a gradual decline in H_2 uptake of *A. glutinosa* nodules over the first 80 minutes of the assay, although they used detached nodules unlike the present study where nodules remained attached to roots. This difference together with the absence of standard errors in their report makes it difficult to comment on their data, particularly in view of the large

variability observed in this study. However, in this study hydrogen uptake, as would be expected from nodules with such high R.E.s was observed for all nodules (Table 31), and is in agreement with previous observations (eg Roelofsen and Akkermans, 1979; Benson, 1979).

The comparisons of strain effectivity in this study all involved comparisons of plant growth over relatively long periods and subsequent analysis of the plant material for nitrogen. This method is, however, both time consuming and destructive. An alternative method using measurements of nitrogenase activity would be particularly advantageous. The general similarity between plots of nitrogenase activity (Figure 13), plant dry weight (Figure 8) and plant nitrogen (Figure 9) to nodule dry weight suggest that such methods may be useful for screening strain effectivity. The reversal of specific activity for some strains, however, (eg 1.1.1[BuC] and 1.1.14[Q]) indicate that such results should be viewed with caution. However, as nitrogenase assays were performed some 3 to 4 weeks after the harvest of plants for analysis of dry matter accretion, further growth probably increased inter-plant competition. It is possible, therefore, that if such competition effects were eliminated then the acetylene reduction assay may provide results broadly comparable with growth analysis.

5.3.0 Utilisation of *Frankia* for inoculation of *Alnus* in field plantings.

The absence of a clear relationship between either site exposure or pH (Table 32) suggests that other soil factors may be responsible for the observed poor nodulation and growth of *A. rubra* on peat soils compared to growth on mineral soils. The observation that peat soils collected from the area surrounding planted alders would not nodulate test seedlings unless inoculated with *Frankia* (Table 33) suggests that prior to planting with *A. rubra* the soils did not contain a viable *Frankia* endophyte. Furthermore, this observation also suggests that *Frankia* was only introduced to the sites studied from infections acquired by plants in the nursery. On plants growing in mineral soils nodules were widespread throughout the root system whereas on plants growing in peats they were always confined to the root crown - where the initial nursery infections would have developed (Table 33). It is clear that either infection or spread of the *Frankia* endophyte through the soil is inhibited or prevented in peats. Previous observations of field nodulation of *Alnus* on peats were made by McVean (1956) who commented on a peat site where *A. glutinosa* was completely devoid of nodules but where nodulation of *Myrica gale* nearby appeared to be normal. This suggests that there are particular problems with the infection of *Alnus spp* in peat since endophyte able to survive in these conditions clearly were present and the endophyte from *Myrica spp* normally nodulates *Alnus spp* (see Introduction). Further it shows clearly how soil type may be an important factor influencing field nodulation and thus growth of *Alnus*.

Further evidence for the effects of soil type on the nodulation and growth of *Alnus* spp. was obtained in a glasshouse study. Species, provenance and inoculum source were all demonstrated to be important determinants of growth (Tables 35 to 43; Figures 16 to 23), thus confirming previous results obtained in nitrogen-free culture (see 4.2.0). Available nitrogen in the soils was not assayed and although total nitrogen analysis suggested that soils were not severely nitrogen deficient (Table 34), inoculation with *Frankia* invariably improved nodulation and growth. Maximum plant growth was obtained by the use of particular sources of *Frankia* and the benefits of inoculation were greatest in Leadburn acid brown earth soil which was of a lower nutrient status (Table 34) . The importance of thoroughly testing the strains prior to large scale inoculation in the field is highlighted by the performance of ArI5 inoculated plants. Previous experiments using ArI5 as inoculum showed this strain to be both highly infective and effective on *A. rubra* grown in nitrogen-free culture (Tables 18, 20, 21 and 46). However, inoculation of plants in this experiment with crushed nodules containing ArI5 alone gave the poorest growth responses and in one case the growth of plants inoculated with ArI5 was not significantly different from the control plants. The possibility that substances released from the host plant cells during crushing may be responsible for poor nodulation with this strain seems unlikely since infection and growth response with the other *Frankia* sources was satisfactory. Also, in separate experiments addition of crushed nodule homogenates to *Frankia* cultures did not reduce their ability to nodulate *A. rubra* (Tables 22 and 23). It is likely that the other sources of crushed nodules contained more than one *Frankia* strain and hence there could be some selection for that strain most adapted to a particular soil. The other crushed nodules

also were at least partly sp^+. Greater infectivity of sp^+ as opposed to sp^- *Frankia* has been observed previously (Houwers and Akkermans, 1981).

Although the growth responses of *Alnus* to inoculation with different sources of *Frankia* were diverse it was possible to make some general observations on the relationship between plant growth and nodule dry weight. In agreement with observations previously made in nitrogen-free culture increased growth of *A. rubra* was generally accompanied in homologous association by increased nodule dry weight or greater nodule productivity, with nodule specific activity remaining relatively constant. In heterologous associations, as observed previously in nitrogen-free culture, differences in growth were due more to differences in nodule specific activity (Figures 22 and 23) with nodule dry weight per plant varying over a much smaller range.

The most striking differences in growth were observed between plants grown on the two mineral soils tested and on peat. Poor growth of alders on acid peat has been observed by many other workers but can be improved by fertiliser application, particularly phosphate (eg McVean; 1956, 1962). Seedlings receiving phosphate also nodulated more readily than the chlorotic, non-fertilised seedlings (McVean, 1962). However, in the present study although application of phosphate improved growth of *Alnus* on the acid brown earth in the second year, growth on peat was not improved significantly. Further work is clearly required to elucidate the precise cause of poor growth of *Alnus* on peat and to elucidate the relative effects on nodulation or on the inhibition of other aspects of plant growth. However, the possible causes of poor survival of *Frankia* were pursued further. It

was shown clearly that aqueous extracts of a mineral soil can stimulate *Frankia* growth *in vitro* and therefore, presumably, contained a stimulatory water-soluble substance(s) (Table 45). The response to aqueous extracts from peat, however, seems to be strain dependant. Growth of a strain isolated from a peat site was either stimulated or unaffected, depending on the source of the peat extract, whereas the growth of a mineral soil isolate was totally inhibited by the peat extracts. Peats apparently contain a substance(s) which may inhibit or at best have no effect on the growth of some *Frankia*, whereas other strains may be able either to tolerate this substance(s) or in some cases be stimulated by it or some other component of the peat extract. That the stimulatory effects of the peat extract were only observed on the growth of the Wauchope strain which was isolated from nodules from the same site as the peat suggests that there may be specific adaptation or selection of strains best adapted to particular soils. However, this type of experiment clearly does not parallel exactly what happens *in situ* since *Frankia* was absent from peat samples taken from areas adjacent to the alders. Nevertheless, the results indicate that the ability of a strain to survive in particular soils should be considered in addition to the potential of a strain for nodulation, nitrogen fixation and its competitive abilities when assessing strains for use as inoculum.

There are no previous reports indicating adaptation of *Frankia* to particular soil conditions although there are reports demonstrating variations in the response of *Frankia* strains to different environmental conditions (see Introduction). Perhaps the greatest variation reported for *Frankia* in culture, however, is the ability of strains to utilise different substrates for growth (see

Introduction). These substrates which include organic and fatty acids occur frequently in soils; the former commonly metabolic products of fungi and the latter of bacteria (Stevenson, 1967). It is possible, therefore, that the growth stimulation observed in this study is due to the presence of such compounds in the extracts and the adaptation of the strain isolated from one of the peat sites to the substrates normally present there, although this would not explain the total inhibition of some strains following the addition of an extract. A further group of soil compounds which might contribute to differential growth responses of particular strains are the humic acids. Visser (1985) reported that the addition of humic acids to selective media at various concentrations stimulated the growth of microorganisms originally isolated from an organic soil, in which humic acids are usually present at higher levels than in more mineral soils. On the addition of the same extracts to media containing microorganisms originally isolated from a sandy soil, however, growth stimulation was observed at low concentrations; at higher concentrations extracts were either less stimulatory or, in the case of proteolytic organisms and humic acids of relatively low molecular weight, inhibitory. In the present study, the extract from mineral soil would probably contain only low levels of humic acids whereas the levels in peat extracts would be higher. If *Frankia* behaved in the same way as the organisms in Visser's study then this could explain the observed results. Further evidence of the inhibitory effects of water-soluble substances in soils has been reported by Jalal and Read (1983). They identified a number of 'free' phenolic and simple fatty acid compounds, in a *Calluna* heathland soil, some of which are known to have strong phytotoxic and specifically fungitoxic properties. A later report discusses evidence which suggests that at low concentrations most of

the phenolic acids can be used as carbon sources by the mycorrhizal fungus *Pezizella ericae*. (Read, 1986).

Further evidence for a possible role of humic acids comes from the demonstration by Shipton and Burggraaf (1982) that vanillic acid will support a low growth rate of *Frankia*. However, the concentrations they used were approximately 18 times greater than those Visser had shown to be inhibitory to some organisms and it is possible that had they used lower concentrations more growth would have been observed. This compound is a constituent of humic acids, and has also been identified in heathland soil extracts by Jalal and Read (1983). Visser also suggests that rather than act as carbon sources humic acids may act like Tweens and through changes in cell wall permeability facilitate the utilisation of compounds not normally used. Furthermore, he concluded that microorganisms from the organic soil seem to be better adapted to high concentrations of humic products in their environment and are able to benefit more from them than organisms from a sandy soil. The observations made in the present study, therefore, appear to show many parallels with Visser's work and indicate the importance of further study of the effect of this group of compounds on the saprophytic growth of *Frankia* and the infection process.

That *Frankia* were unable to grow in any of the extracts alone, even when a suitable carbon source had been added suggests limitation by other factors, although it is not possible to determine which from the results.

Another important factor affecting the success of field inoculation is strain competition. Studies of competition between *Frankia* for host nodulation necessarily involve the use of a stable marker or characteristic, so that the source of infection from which nodules develop can be recognised. Methods which could prove useful in such studies are antibiotic resistance or protein patterns. However, the technology necessary for the former has not yet been developed for *Frankia* and the latter technique would be unsuitable for large experiments due to the time involved in the assay. In this study the possibility of using *in vivo* spore production as an easily recognisable morphological marker was explored. In this experiment, as in previous experiments discussed in 4.2.2, the spore type of the nodules which developed following inoculation and maintenance of plants under identical conditions in Perlite was identical to the source inoculum, either sp^+ or sp^-, (see 3.3.3). This adds further support for the involvement of genetic rather than environmental factors in sporangial development *in vivo*. However, the same result would have been obtained if expression of *in vivo* sporangial development by different strains were influenced by different factors or by the same factors to a different extent with different strains. Clearly it is possible to use this data to support either hypothesis although the balance is perhaps in favour of the 'genetic' rather than the 'environmental' hypothesis when considered together with the previous observations.

In order to assess competition between sp^+ and sp^- strains plants inoculated with sp- strains were planted out into plots containing soil which normally gave rise to predominantly sp^+ nodules, the soil watered with sp^- inoculum and the spore type of the nodules produced

after 20 months of growth assessed. Comparison with plants inoculated with sp^+ inoculum and planted into similar soil beds which were not watered with sp^- inoculum enabled assessment of the extent to which the introduced sp^- strain would compete with the indigenous *Frankia*(e) for formation of new nodules. Although sp^+ nodules did occur on sp^- inoculated plants (Table 47), their occurrence was considerably reduced by inoculation of the soil with sp^- *Frankia* which must, therefore, have competed successfully for infection of new *A. rubra* roots with strains already present in the soil. These results confirm previous work by Dijk (1984) who conducted experiments in water culture and concluded that competition for infection was not complicated by strong antagonistic effects between sp^+ and sp^- strains.

Although the benefits of symbiotic nitrogen fixation by actinorhizal plants have long been recognised and the variation in potential of symbiotic nitrogen fixation by different *Frankia* strains has been demonstrated (see Introduction), there have been very few studies to compare the effects on plant nodulation and growth of inoculation with different *Frankia* strains in the field. The evidence already presented in this Thesis and discussed earlier in this section and elsewhere (see Introduction), demonstrating the effects of edaphic factors on nodulation and nitrogen fixation, indicate that results obtained in the field may not always parallel those in the laboratory.

The field studies reported, however, do indicate that inoculation can improve yield. Gauthier *et al.* (1985) demonstrated that yield of *Casuarina* grown in sterilised soil and inoculated with *Frankia* was improved, although as only one *Frankia* strain was used comparisons

between strains were not possible. Perinet *et al.* (1985) compared a variety of methods for the inoculation of a large number of actinorhizal seedlings in the nursery with *Frankia* isolates from a number of species and showed that such large scale inoculation was feasible but did not discuss *Frankia* effectivity.

The observed differences in dry weight and nitrogen accretion between inoculated and uninoculated plants in this study clearly demonstrate the benefits of nursery inoculation (Tables 48 to 50). Although nodules did also occur on the uninoculated plants their peripheral position and low dry weight per plant indicates that they would only have formed relatively late in the growth period. Such infections, therefore, would have only made a limited contribution to the nitrogen nutrition of the plants. Outside the nursery beds, however, plants are always nodulated and so the endophyte is normally present in non-sterilised soil in the nursery. The chemical sterilisation procedure carried out prior to the sowing of fruits clearly destroys most of the endophyte present so that nodulation must, without inoculation, normally be very limited as observed in the present study. The effectiveness of soil sterilisation for eliminating soil-borne *Frankia* has also been observed at other nurseries (M. Hollingsworth, personal communication). Inoculation with *Frankia* at the nursery stage should obviously be advantageous for growth unless replaced by adequate fertiliser. In this study the growth of plants was so improved following inoculation that seedlings were ready for outplanting in 1 year instead of the usual 2 or even 3 years.

Interestingly the root to shoot ratios of plants inoculated with ArI4 or crushed nodules were much lower than when inoculated with

1.2.5[Q](b), which gave ratios similar to uninoculated plants. Previously in this Thesis it has not been possible to ascribe differences in root and shoot ratios of inoculated plants to strain effects as, in general, these were accompanied by differences in growth which have been reported to affect ratios (Evans 1972). However, in this study strain clearly affected the ratio. Clearly different allocations of dry matter between root and shoot may be an important criterion upon which selections of strain for optimal symbioses may be made since such differences could have significant effects on nutrient cycling. In the present study, however, it is conceivable that such ratios may change during growth over longer periods.

Although the benefits of inoculation are clear, it is interesting to compare further the symbiotic performance of the strains in the nursery with their performance under more controlled conditions on plants cultured in nitrogen-free Perlite. In the nursery, inoculation improved plant growth above that of fertilised but uninoculated controls. However, the growth of plants inoculated with different *Frankia* was not significantly different from each other (Table 48). In contrast, in previous experiments in Perlite there was a clear difference, the growth of plants inoculated with 1.2.5[Q](b) being superior to ArI4, with crushed nodule inoculated plants showing poorest growth (Table 20). This order of effectivity of strains was apparently maintained in the nursery although variation between plants now masked most of the much smaller growth differences with different strains. Comparison of the nodule dw to plant nitrogen ratios in the nursery with those observed in Perlite also revealed the same relative

order between plants inoculated with different strains (Table 50). In Perlite this corresponds to the nodule specific activity for nitrogen fixation but in the nursery the availability for plant growth of soil mineral nitrogen makes it impossible to determine the nodule specific activity absolutely. It is interesting nevertheless that estimates of this ratio in the field obtained by subtracting the nitrogen content of uninoculated plants from inoculated plants (Table 50) are only around one half that in Perlite grown plants. It is possible that this may reflect lower temperatures in soil in the field than in the growth cabinets, giving rise to lower rates of nitrogen fixation per unit nodule weight. The order and comparative size of differences in this ratio between plants inoculated with different strains and grown in the nursery is similar to that in the field. It is clear, therefore, that differences in nodule productivity per plant must be the main cause of the differences in effectivity of strains in nursery and Perlite grown plants. The major difference was a dramatic increase in the weight of nodules formed on crushed nodule-inoculated plants in the nursery, where nodule dry weights were similar to those on 1.2.5[Q](b) inoculated plants. In Perlite, nodule dry weights per plant of the former plants were only about one third of the latter. Differences in infectivity were also observed, with nodules distributed differently in the two growth media. Nodules formed after inoculation with crushed nodules and ArI4 were distributed throughout the root system in the nursery, in contrast to growth in Perlite where they were restricted to the root crown. These observations taken together suggest that infection and spread of the endophyte was restricted in Perlite. Further support for this conclusion comes from the observation that only 83% (Table 19) and 23% (Table 22) of plants inoculated with crushed nodules were nodulated at harvest in

Perlite whereas in the nursery 100% of plants were nodulated. Clearly, therefore, although growth in nitrogen-free Perlite culture may give an indication of the relative nodule specific activity for nitrogen fixation of different *Frankia*, the relative effectivity of symbioses in the field may well be different due to soil and other environmental effects on infectivity and nodule growth.

This study, therefore, has provided an analysis of several aspects of the *Frankia/Alnus* symbiosis. That the endophyte can be isolated reliably from nodules from many sites was demonstrated unequivocally. However, the failure to isolate from sp^+ nodules *Frankia* which form sp^+ nodules on test plants clearly requires further investigation in the future. The strains that were isolated varied both in their nutrition *in vitro* and in their effectivity in symbiosis. Important was the finding that the North American *Frankia* strains were no more and in some cases were less effective than indigenous British strains. Perhaps most interesting was the indication that in homologous associations, nodule specific activity was relatively constant and that differences in nodule growth contributed more to variations in effectivity whereas in heterologous associations nodule growth was relatively constant and variations in effectivity were due more to changes in nodule specific activity. Not surprisingly, the growth of *Alnus* was shown to be influenced by the soil in which it was grown with growth in peat particularly poor. Soil type was also clearly shown to affect strain effectivity and its determinants. Particularly interesting was the suggestion that *Frankia* strains may be adapted to specific soil components. Finally, and of greatest practical importance were the demonstrations of improved growth of plants in the nursery through inoculation with *Frankia*. Further experiments are

required to extend these findings if the aim of encouraging nurserymen to inoculate *Alnus* with different strains of *Frankia* is to be realised fully.

Appendix 1

Composition of *Frankia* isolation and growth medium. Author(s) in brackets indicates the report in which similar medium first appeared.

EMC+P (Benson, 1982)

Component	Concentration gl^{-1}			
K_2HPO_4	3.0	*		
KH_2PO4	2.0	*		
$MgSO_4.7H_2O$	0.2			
NaCl			0.3	
FeNaEDTA			0.16	*
Micronutrient salts (as in FB)				
Vitamin mixture (Tjepkema *et al.*1980)			1ml	
Sodium puruvate (filter sterilised)				3.0
Casamino acids (Difco)				3.0
Agar (if required)				8.0

Adjust pH to 6.8 with HCl or NaOH.

* pH and autoclave together but separate from rest of the medium.

Vitamin mixture	mg/100ml
Thiamine HCl	10
Nicotinic acid	50
Pyridoxine HCl	50
Succinic acid	1.2g/100ml.

Qmod (Lalonde and Calvert, 1979)

Component	Concentration gl^{-1}	
K_2HPO_4	0.3	*
$NaH_2PO_4.2H_2O$	0.2	*
$MgSO_4.7H_2O$	0.2	
KCl	0.2	
Yeast extract (Difco)	0.5	
Bacto-peptone (Difco)	5.0	
Glucose	10.0	
(filter sterilise with 0.001g cycloheximide)		
Ferric citrate (1% in 1% citric acid)	1ml	
Minor salts **	1ml	
Agar (if required)	8.0	

Adjust pH to 6.8 with HCl or NaOH. Then add:-

$CaCO_3$	0.1
Lipid supplement ***	$2.5mgl^{-1}$

* pH and autoclave together but separate from rest of medium.

** Minor salts	gl^{-1}
H_3BO_3	1.5
$MnSO_4.7H_2O$	0.8
$ZnSO4.7H_2O$	0.6
$CuSO_4$ $7H_2O$	0.1

Component	Concentration gl^{-1}
$(NH_4)_6MO_7O_{24}.4H_2O$	0.2
$CoSO_4.7H_2O$	0.1

*** Lipid supplement: dissolve 500mg of L-lecithin (commercial grade from soybeans, 22% phosphatidyl choline, P-5638 from Sigma Chemical Co.) in 50ml absolute ethanol, and add 50ml distilled water).

Bu, BuC and BuCT (Shipton and Burggraaf, 1982)

Bu

Component	Concentration gl^{-1}	
$CaCl_2.2H_2O$	0.1	
$MgSO_4.7H_2O$	0.2	
NH_4NO_3	0.1	
FeNaEDTA	0.01	*
Biotin	0.002	
K_2HPO_4	1.0	*
$NaH_2PO_4.2H_2O$	0.67	*
Sodium propionate	0.5	
Micronutrient salts (as in FB)		
Agar (if required)	8.0	

Adjust pH to 6.8 with HCl or NaOH.

* pH and autoclave together but separate from rest of medium.

BuC - as Bu with the addition of $1gl^{-1}$ Casamino acids (filter sterilised).

BuCT - as BuC with the addition of $0.5gl^{-1}$ Tween 80.

P(-) and P(+) (Pommer, 1959)

P(-)

Component	Concentration gl^{-1}	
Glucose	10.0	
(filter sterilised with 0.005g cyclohexamide)		
Asparagine	0.5	
K_2HPO_4	0.5	*
Micronutrient salts (as in FB)	1ml	
Agar (if required)	8.0	

Adjust pH to 6.8 with HCL or NaOH.

* pH and autoclave separately.

P(+) - as P(-) with the addition of an aqueous extract of 3.0g nodules. Prepared by homogenising washed and surface sterilised (10% bleach) nodules and straining through muslin.

M-N/Cas, M-N/Cas/TL, P-N/Cas, P-N/Cas/TL.

M-N/Cas - BuC without NH_4Cl.

M-N/Cas/TL - M-N/Cas with the addition of a Total Lipid (TL) extract prepared as described in Burggraaf (1984)

P-N/Cas - BuC without sodium propionate.

P-N/Cas/TL - P-N/Cas with the addition of a TL extract prepared as described in Burggraaf (1984).

FB (Baker and Torrey, 1979)

Component	Concentration	gl^{-1}
Yeast extract (Difco)		5.0
Dextrose (Difco)		10.0
Casamino acids (Difco 0		5.0
Vitamin B_{12}	0.0016	
Micronutrient salts		mgl^{-1}
H_3BO_3		1.5
$ZnSO_4.7H_2O$	1.5	
$MnSO_4.H_2O$		4.5
$NaMoO_4.H_2O$	0.25	
$CuSO_{4.5H2}O$	0.04	
Agar (if required)		$8.0gl^{-1}$

pH adjusted to 6.4 with HCl or NaOH.

Appendix 2

Acronyms used to designate *Frankia* strains in this study.

There are two components to the acronym. The first part identifies the host plant species and the individual isolation and is made up of 3 numbers each separated by a decimal. The first number indicates the genus - 1, *Alnus*; 2 - *Hippophae*. The second indicates the species - 1.1, *Alnus glutinosa*, 1.2, *A. rubra*; 1.3, *A. sinuata*; 1.4, *A. incana* and 2.1 *Hippophae rhamnoides*. The third number indicates the isolation medium upon which the strain was first isolated. Thus 1.1.14[Q] is a strain isolated from *A. glutinosa* on Q medium during isolation attempt number 14. Several strains have now been assigned designates according to the classification scheme of Lechevalier (1983). These strains, together with their designates, are listed in Hooker and Wheeler (1987).

Plant nutrients

Crones-N (Bond, 1950)

Component	g
KCL	375
$CaSO_4 . 2H_2O$	250
$MgSO_4 . 7H_2O$	250
$Ca(PO_4)_2$	125
$Fe_3(PO_4)_2 . 8H_2O$	125

Full strength: $2.25 gl^{-1}$

Hoaglands A-Z micronutrients.

Component	Concentration gl^{-1}
H_3BO_3	2.86
$MnCl_2 . 4H_2O$	1.81
$ZnSO4 . 7H_2O$	0.22
$CuSO_4 . 5H_2O$	0.08
$NaMoO_4 . 2H_2O$	0.025

Liquinure

Composition	%
Nitrogen	8.0
Phosphorus	1.7
Potassium	3.3
	$mgkg^{-1}$
Magnesium	200.00
Manganese	0.33
Boron	3.30
Zinc	66.00
Copper	10.00
Molybdenum	3.30

Ammonium phosphate is produced *in situ* by the reaction of phosphoric acid and potassium hydroxide.

Table 16S: Analysis of Variance of growth parameters of *A. rubra* inoculated with crushed nodule inocula.

Plant d.w.					
DUE TO	DF	SS	MS	F	
Factor	2	144.5	72.2	7.17	**
Error	21	211.6	10.1		
Total	23	356.1			
Nodule d.w.					
DUE TO	DF	SS	MS	F	
Factor	2	0.04380	0.02190	5.36	*
Error	21	0.08577	0.00408		
Total	23	0.12958			
Nodule no.					
DUE TO	DF	SS	MS	F	
Factor	2	97416	48708	37.12	***
Error	21	27559	1312		
Total	23	124976			
Ratio root to shoot d.w.					
DUE TO	DF	SS	MS	F	
Factor	2	0.154	0.077	4.053	*
Error	21	0.390	0.019		
Total	23	0.544			
Plant / nodule d.w.					
DUE TO	DF	SS	MS	F	
Factor	2	15312	7656	46.52	***
Error	21	3456	165		
Total	23	18768			

Table 18S: Analysis of Variance of dry weights of *A.rubra* inoculated with *Frankia* and grown in water culture.

DUE TO	DF	SS	MS	F	
Factor	4	26.83	6.71	7.54	***
Error	59	52.29	0.89		
Total	63	79.12			

Table 20S : Analysis of Variance of growth parameters of *A. rubra* inoculated with *Frankia* strains (Batches 1 to 3).

Batch 1

Plant d.w.

DUE TO	DF	SS	MS	F	
Factor	6	74.31	12.38	5.93	***
Error	75	156.52	2.09		
Total	81	230.82			

Nodule d.w.

DUE TO	DF	SS	MS	F	
Factor	6	0.04751	0.00792	7.20	***
Error	75	0.08798	0.0011		
Total	81	0.13548			

Table 20S continued

Nodule no.

DUE TO	DF	SS	MS	F	
Factor	6	11607.2	1934.5	30.60	***
Error	75	4741.3	63.2		
Total	81	16348.6			

Ratio root to shoot d.w.

DUE TO	DF	SS	MS	F	
Factor	6	0.11824	0.01971	2.10	NS
Error	75	0.70513	0.00940		
Total	81	0.82337			

Plant / nodule d.w.

DUE TO	DF	SS	MS	F	
Factor	6	7612	1269	12.12	***
Error	75	7853	105		
Total	81	15464			

Batch 2

Plant d.w.

DUE TO	DF	SS	MS	F	
Factor	7	2.85.37	40.77	4.70	***
Error	88	762.59	8.67		
Total	95	1047.95			

Nodule d.w.

DUE TO	DF	SS	MS	F	
Factor	7	0.00834	0.00119	0.23	NS
Error	88	0.45449	0.00516		
Total	95	0.46283			

Table 20S continued

Nodule no.

DUE TO	DF	SS	MS	F	
Factor	7	86843	12406	43.87	***
Error	88	24887	283		
Total	95	111730			

Ratio root to shoot d.w.

DUE TO	DF	SS	MS	F	
Factor	7	0.0404	0.0058	0.35	NS
Error	88	1.4538	0.0165		
Total	95	1.4942			

Plant / nodule d.w.

DUE TO	DF	SS	MS	F	
Factor	7	29585	4226	33.42	***
Error	88	11129	126		
Total	95	40714			

Batch 3

Plant d.w.

DUE TO	DF	SS	MS	F	
Factor	7	73.85	10.55	5.68	***
Error	88	163.38	1.86		
Total	95	237.23			

Nodule d.w.

DUE TO	DF	SS	MS	F	
Factor	7	0.013109	0.001873	2.86	*
Error	88	0.057597	0.000655		
Total	95	0.070706			

Table 20S continued

Nodule no.

DUE TO	DF	SS	MS	F	
Factor	7	10669.8	1524.3	28.33	***
Error	88	4737.7	53.8		
Total	95	15407.5			

Ratio root to shoot d.w.

DUE TO	DF	SS	MS	F	
Factor	7	0.10224	0.01461	4.35	***
Error	88	0.29526	0.00336		
Total	95	0.39750			

Plant / nodule d.w.

DUE TO	DF	SS	MS	F	
Factor	7	13983	1998	8.99	***
Error	88	19544	222		
Total	95	33527			

Table 23S: Analysis of Variance of growth parameters of *A. rubra* inoculated with *Frankia* strains (Batch 4).

Plant d.w.

DUE TO	DF	SS	MS	F	
Factor	2	14.42	7.21	1.02	NS
Error	28	197.75	7.06		
Total	30	212.17			

Table 23S continued

Nodule d.w.

DUE TO	DF	SS	MS	F	
Factor	2	0.01199	0.00599	1.87	NS
Error	28	0.08989	0.00321		
Total	30	0.10188			

Nodule no.

DUE TO	DF	SS	MS	F	
Factor	2	4072	2036	12.61	***
Error	28	4520	161		
Total	30	8592			

Ratio root to shoot d.w.

DUE TO	DF	SS	MS	F	
Factor	2	0.18519	0.09259	11.29	***
Error	28	0.24703	0.0082		
Total	30	0.43222			

Plant / nodule d.w.

DUE TO	DF	SS	MS	F
Factor	2	354	177	1.07
Error	28	3251	166	
Total	30	3605		

Table 25S: Analysis of Variance of growth parameters of *A. rubra* and *A. glutinosa* inoculated with *Frankia* strains (Batch 5).

(a) One Factor

A. rubra

Plant d.w.

DUE TO	DF	SS	MS	F	
Factor	2	5.462	2.731	6.48	**
Error	33	13.899	0.421		
Total	35	19.361			

Nodule d.w.

DUE TO	DF	SS	MS	F	
Factor	2	0.000283	0.000142	0.53	***
Error	33	0.00812	0.000267		
Total	35	0.009096			

Nodule no.

DUE TO	DF	SS	MS	F	
Factor	2	181.1	90.5	2.15	NS
Error	33	1386.6	42.0		
Total	35	1567.6			

Ratio root to shoot d.w.

DUE TO	DF	SS	MS	F	
Factor	2	0.05278	0.02639	5.98	**
Error	33	0.14551	0.00441		
Total	35	0.19829			

Table 25S continued

Plant / nodule d.w.

DUE TO	DF	SS	MS	F	
Factor	2	4262.1	2131.1	33.90	***
Error	33	2074.7	62.9		
Total	35	6336.8			

A. glutinosa

Plant d.w.

DUE TO	DF	SS	MS	F	
Factor	2	20.45	10.23	4.59	**
Error	33	73.46	2.23		
Total	35	93.91			

Nodule d.w.

DUE TO	DF	SS	MS	F	
Factor	2	0.009644	0.004822	5.74	**
Error	33	0.027721	0.000840		
Total	35	0.037365			

Nodule no.

DUE TO	DF	SS	MS	F	
Factor	2	2098	1049	6.08	**
Error	33	5691	172		
Total	35	7789			

Ratio root to shoot d.w.

DUE TO	DF	SS	MS	F	
Factor	2	0.02932	0.01466	3.48	*
Error	33	0.13895	0.00421		
Total	35	0.16827			

Table 25S continued

Plant / nodule d.w.

DUE TO	DF	SS	MS	F	
Factor	2	911	456	2.72	NS
Error	33	5522	167		
Total	35	6434			

(b) Two Factor.

Plant d.w.

DUE TO	DF	SS	MS	F	
Strain	2	10.884	5.442	4.111	*
Species	1	111.253	111.253	84.051	***
Strain.Species	2	15.029	7.515	5.677	**
Residual	66	87.360	1.324		
Total	71	224.527	3.162		

Nodule d.w.

DUE TO	DF	SS	MS	F	
Strain	2	0.0053117	0.0026558	4.798	*
Species	1	0.0180500	0.018050	32.609	***
Strain.Species	2	0.0046157	0.0023079	4.169	*
Residual	66	0.0365331	0.0005535		
Total	71	0.0645105	0.0009086		

Table 25S continued

Nodule no.

DUE TO	DF	SS	MS	F	
Strain	2	1494.7	747.3	6.970	**
Species	1	7280.2	7280.2	67.894	***
Strain.Species	2	784.5	392.3	3.658	*
Residual	66	7077.2	107.2		
Total	71	16636.6	234.3		

Ratio root to shoot d.w.

DUE TO	DF	SS	MS	F	
Strain	2	0.028797	0.014398	3.341	*
Species	1	0.032909	0.032909	7.635	**
Strain. Species	2	0.053302	0.026651	6.184	**
Residual	66	0.284460	0.004310		
Total	71	0.399467	0.005626		

Table 25S continued

Plant / nodule d.w.

DUE TO	DF	SS	MS	F	
Strain	2	952.7	476.3	4.138	*
Species	1	21442.4	21442.4	186.285	***
Strain.Species	2	4220.9	2110.5	18.335	***
Residual	66	7597.0	115.1		
Total	71	34213.0	481.9		

Table 28S: Analysis of Variance of growth parameters of *A. rubra from a number of seed sources after inoculation with Frankia* strains (Batch 6).

McNab's Farm

Plant d.w.

DUE TO	DF	SS	MS	F	
Factor	2	1.09	0.54	0.43	NS
Error	33	42.00	1.27		
Total	35	43.08			

Table 28S continued

Nodule d.w.

DUE TO	DF	SS	MS	F	
Factor	2	0.002085	0.001043	1.13	NS
Error	33	0.030469	0.000923		
Total	35	0.032555			

Nodule no.

DUE TO	DF	SS	MS	F	
Factor	2	35.4	17.7	1.07	NS
Error	33	546.2	16.6		
Total	35	581.6			

Ratio root to shoot d.w.

DUE TO	DF	SS	MS	F	
Factor	2	0.04619	0.02310	3.80	*
Error	33	0.20059	0.00608		
Total	35	0.24678			

Plant / nodule d.w.

DUE TO	DF	SS	MS	F	
Factor	2	1.09	0.54	0.43	NS
Error	33	42.00	1.27		
Total	35	43.08			

Menzies Bay

Plant d.w.

DUE TO	DF	SS	MS	F	
Factor	2	7.63	3.81	1.33	NS
Error	33	94.53	2.86		
Total	35	102.16			

Table 28S continued

Nodule d.w.

DUE TO	DF	SS	MS	F	
Factor	2	0.005691	0.002846	3.70	*
Error	33	0.025372	0.000769		
Total	35	0.031063			

Nodule no.

DUE TO	DF	SS	MS	F	
Factor	2	360.5	180.2	16.45	***
Error	33	361.5	11.0		
Total	35	722.0			

Ratio root to shoot d.w.

DUE TO	DF	SS	MS	F	
Factor	2	0.01971	0.00985	1.71	NS
Error	33	0.18986	0.00575		
Total	35	0.20957			

Plant / nodule d.w.

DUE TO	DF	SS	MS	F	
Factor	2	1818	909	6.28	**
Error	33	4779	145		
Total	35	6597			

Table 28S continued

Prince Rupert

Plant d.w.

DUE TO	DF	SS	MS	F	
Factor	2	7.35	3.68	3.11	NS
Error	33	39.00	1.18		
Total	35	46.36			

Nodule d.w.

DUE TO	DF	SS	MS	F	
Factor	2	0.003069	0.001535	3.67	*
Error	33	0.013797	0.000418		
Total	35	0.016867			

Nodule no.

DUE TO	DF	SS	MS	F	
Factor	2	10.7	5.4	0.22	NS
Error	33	820.2	24.9		
Total	35	831.0			

Ratio root to shoot d.w.

DUE TO	DF	SS	MS	F	
Factor	2	0.00648	0.00324	1.36	NS
Error	33	0.07887	0.00239		
Total	35	0.08535			

Plant / nodule d.w.

DUE TO	DF	SS	MS	F	
Factor	2	490	245	0.36	NS
Error	33	22373	678		
Total	35	22862			

Table 28S continued

(b) Two Factor

Plant d.w.

DUE TO	DF	SS	MS	F	
Strain	2	9.690	4.845	2.733	NS
Provenance	2	8.320	4.160	2.346	NS
Strain. Provenance	4	6.378	1.595	0.899	NS
Residual	99	175.531	1.773		
Total	107	199.919	1.868		

Nodule d.w.

DUE TO	DF	SS	MS	F	
Strain	2	0.0038994	0.0019497	2.772	NS
Provenance	2	0.0023135	0.0011567	1.644	NS
Strain. Provenance	4	0.0069468	0.0017367	2.469	NS
Residual	99	0.0696384	0.0007034		
Total	107	0.0827981	0.0007738		

Nodule no.

DUE TO	DF	SS	MS	F	
Strain	2	105.57	52.79	3.024	NS
Provenance	2	38.02	19.01	1.089	NS
Strain. Provenance	4	301.04	75.26	4.312	**
Residual	99	1727.92	17.45		
Total	107	2172.54	20.30		

Table 28S continued

Ratio root to shoot d.w.

DUE TO	DF	SS	MS	F	
Strain	2	0.021661	0.010830	2.285	NS
Provenance	2	0.026950	0.013475	2.843	NS
Strain. Provenance	4	0.050716	0.0126679	2.675	*
Residual	99	0.469320	0.004741		
Total	107	0.568647	0.005314		

Plant / nodule d.w.

DUE TO	DF	SS	MS	F	
Strain	2	426.2	213.1	0.624	NS
Provenance	2	1077.5	538.8	1.579	NS
Strain. Provenance	4	2086.6	521.6	1.529	NS
Residual	99	33781.3	341.2		
Total	107	37371.5	349.3		

Table 48S: Analysis of Variance of growth parameters of inoculated and uninoculated *A. rubra* grown in a nursery.

Plant d.w.

DUE TO	DF	SS	MS	F	
Factor	4	88.621	22.155	26.56	***
Error	95	79.247	0.834		
Total	99	167.867			

Table 48S continued

Ratio of root to shoot d.w.

DUE TO	DF	SS	MS	F	
Factor	4	0.29753	0.07438	7.97	***
Error	95	0.88697	0.00934		
Total	99	1.18450			

Nodule d.w.

DUE TO	DF	SS	MS	F	
Factor	4	0.59755	0.14939	34.56	***
Error	95	0.41069	0.00432		
Total	99	1.00824			

Nodule no.

DUE TO	DF	SS	MS	F	
Factor	4	63506	15876	95.23	***
Error	95	15837	167		
Total	99	79343			

References

Akkermans, A. D. L. 1971. Nitrogen fixation and nodulation of *Alnus* and *Hippophae* under natural conditions. PhD thesis. State University, Leiden, The Netherlands.

Akkermans, A. D. L. 1982. Biology of actinorhizal plants and their application in forestry. The Second National Symposium on Biological Nitrogen Fixation, Helsinki, 8-10th June 1982. pp. 261-272. The Finnish National Fund for Research and Development, Helsinki.

Akkermans, A. D. L. and Dijk, C. van. 1981. Non-leguminous root nodule symbioses with actinomycetes and *Rhizobium*. In *Nitrogen Fixation*. ed. Broughton, W. J. Vol. I. pp. 57-103. Oxford University Press.

Akkermans, A. D. L., Roelofsen, W., Blom, J., Huss-Danell, K. and Harkink, R. 1983. Utilisation of carbon and nitrogen compounds by *Frankia* in synthetic media and in root nodules of *Alnus glutinosa*, *Hippophae rhamnoides* and *Datisca cannabina*. *Can. J. Bot.* **61**, 2793-2800.

Baker, D. 1982. A cumulative listing of isolated *Frankiae*, the symbiotic, nitrogen-fixing actinomycetes. In *The Actinomycetes* **17**, 35-42.

Baker, D., Kidd, G. H. and Torrey, J. G. 1979. Separation of actinomycete nodule endophytes from crushed nodule suspensions by Sephadex fractionation. *Bot. Gaz.* **140S** S49-S51.

Baker, D. and O'Keefe, D. 1984. A modified sucrose fractionation procedure for the isolation of Frankiae from actinorhizal root nodules and soil samples. *Pl. Soil*, **78**, 23-28.

Baker, D., Newcomb, W. and Torrey J. G. 1980. Characterisation of an ineffective actinorhizal microsymbiont, *Frankia* sp. EuI1 (Actinomycetales). *Can. J. Microbiol.* **26**, 1072-1089.

Baker, D. and Torrey, J. G. 1979. The isolation and cultivation of actinomycetous root nodule endophytes. In *Symbiotic nitrogen fixation in the management of temperate forests*, ed. Gordon, J. C., Wheeler, C. T. and Perry, D. A., pp.38-57. School of Forestry, Oregon State University, U.S.A.

Baker, D. and Torrey, J.G. 1980. Characterisation of an effective actinorhizal microsymbiont, *Frankia* sp. AvCI1 (Actinomycetales). *Can. J. Microbiol.* **26** 1066-1071.

Barrow, N. J. 1970. Comparison of the adsorption of molybdate, sulphate and phosphate by soils. *Soil Sci.* **109**, 282-288.

Becking, J. H. 1961. A requirement of molybdenum for the symbiotic nitrogen fixation in alder. *Pl. Soil* **15**, 217-227.

Becking, J. H. 1970. Frankiaceae Fam. nov. (Actinomycetales) with one new combination and six new species of the genus *Frankia* Brunchorst 1886.,174. *Int. J. Syst. Bact.***20**, 201.

Becking, J. H. 1974. Frankiaceae (Becking 1970). In *Bergey's Manual of Determinative Bacteriology*. 8th edition. ed. Buchanan, R. E. and Gibbons, N. E. pp 701-706. Williams and Wilkins, Baltimore.

Becking, J. H. 1977. Nitrogen-fixing associations in higher plants other than legumes. In *A treatise on dinitrogen fixation vol 2* ed. Hardy, R. W. F. and Silver, W. S. pp. 185-275. John Wiley & Sons, New York.

Becking, J. H. 1981. The genus *Frankia*. In *The Prokaryotes: A handbook on habitats, isolation and identification of bacteria*, ed. Star, M.P. . 1991-2003. Springer-Verlag, Berlin, West Germany, New York.

Becking, J. H., Boer, De, W. E. and Houwink, A. L. 1964. Electron microscopy of the endophyte of *Alnus glutinosa*. *Antonie van Leeuwenhoek*, **30**, 343.

Benson, D. R. 1982. Isolation of *Frankia* strains from alder actinorhizal root nodules. *Appl. Env. Microbiol.* **28**, 461-465.

Benson, D. R., Arp, D. J. and Burris, R. H. 1979. Cell-free nitrogenase and hydrogenase from actinorhizal root nodules. *Science* **205**, 688-689.

Benson, D. R. and Hanna, D. 1983. *Frankia* diversity in an alder stand as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of whole-cell proteins. *Can. J. Bot.* **61**, 2919-2923.

Berg, R. H. 1983. Preliminary evidence for the involvement of suberization in infection of *Casuarina*. *Can. J. Bot.* **61**, 2910-2918.

Bergesen, F. J. 1970. The quantitative relationship between nitrogen fixation and the acetylene reduction assay. *Aust. J. Biol. Sci.* **23**, 1015-1025.

Berry, A. and Torrey, J. G. 1979. Isolation and characterisation *in vivo* and *in vitro* of an actinomycetous endophyte from *Alnus rubra* Bong. In *Symbiotic nitrogen fixation in the management of temperate forests*, ed. Gordon, J. C., Wheeler, C. T. and Perry, D. A., pp.69-83. School of Forestry, Oregon State University, U.S.A.

Bethnelfalvay, G. J. and Phillips, D. A. 1977. Ontogenetic interactions between photosynthesis and symbiotic nitrogen fixation in legumes. *Plant Physiol.* **60**, 419-421.

Binkley, D., Sollins, P. and McGill, W. B. 1985. Natural abundance of nitrogen-15 as a tool for tracing alder-fixed nitrogen. *Soil Sci. Soc. Am. J.* **49**, 444-447.

Blom, J. and Harkink, R. 1981. Metabolic pathways for gluconeogenesis and energy generation in *Frankia* AvcI1. *Fems Microbiol. Letts.* **11**, 221-224.

Bond, G. 1963. Symbiotic Associations. In *Symp. Soc. Gen. Microbiol.*13. ed. Nutman, P. S. and Mosse, B. pp 72-91.

Bond, G. 1967. Fixation of nitrogen by higher plants other than legumes. *Ann. Rev. Pl. Physiol.* **18**, 107-126.

Bond, G. 1976. The results of the I.B.P. survey of root nodule formation in non-leguminous angiosperms. In *Symbiotic Nitrogen Fixation in Plants.* **Vol. 7.** pp. 443-474. International Biological Programme.Cambridge University Press, Cambridge.

Bond, G. 1983. Taxonomy and distribution of non-legume nitrogen-fixing systems. In *Biological Nitrogen Fixation in Forest Ecosystems: Foundations and Applications.* ed. Gordon, J. C. and Wheeler, C. T. , pp. 55-88. Martinus Nijhoff / Dr. W. Junk Publishers, The Hague.

Bond, G., Adams, J. F. and Kennedy, E. H. 1965. Vitamin B12 analogues in non-legume root nodules. *Nature* **207**, 319-320.

Bond, G.and Hewitt, E. J. 1961. Molybdenum and the fixation of nitrogen in *Myrica gale* root nodules. *Nature* **109**, 1033-1034.

Bond, G and Hewitt, E. J. 1962. Cobalt and the fixation of nitrogen by root nodules of *Alnus* and *Casuarina*. *Nature (London)* **195**, 94-95.

Bond, G., MacConnell, J. and McCallum, A. H. 1956. The nitrogen nutrition of *Hippophae rhamnoides* L. *Ann. Bot.* **20**, 501-512.

Bond, G. and MacKintosh, A. H. 1975. Effect of nitrate-nitrogen on the nodule symbioses of *Coriaria* and *Hippophae*. *Proc. Roy. Soc. Lond. B.* **190**, 199-209.

Bostid. 1984. Casuarinas: nitrogen fixing trees for adverse sites. National Acadamy Press, Washington D.C. pp.118.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein dye binding. *Analytical Biochem.* **72**, 246-254.

Bulen, W. A., Burns, R. C. and LeComte, J. R. 1965. Nitrogen fixation: hydrosulfite as electron donor with cell-free preparations of *Azotobacter vinelandi* and *Rhodospirillum rubrum*. *Proc. Nat. Acad. Sci. USA.* **53**, 532-539.

Burggraaf, A. J. P. 1984. Isolation, cultivation and characterisation of *Frankia* strains from actinorhizal root nodules. PhD thesis. State University, Leiden, The Netherlands. pp. 177.

Burggraaf, A. J. P., Quispel, A., Tak, T. and Valstar, J. 1981. Methods of isolation and cultivation of *Frankia* species from actinorhizas. *Pl. Soil.* **61** , 157-168.

Burggraaf, A. J. P. and Shipton, W. A. 1983. Studies on the growth of *Frankia* isolates in relation to infectivity and nitrogen fixation (acetylene reduction). *Can. J. Bot.* **61**, 2774-2782.

Burggraaf, A. J. P. and Valstar, J. 1984. Heterogeneity within *Frankia* sp. LDAgp1 studied among clones and reisolates. *Pl. Soil* **78**, 29-43.

Burns, R. C. and Hardy, R. W. F. 1975. *Nitrogen fixation in Bacteria and Higher Plants.* Springer Verlag, New York.

Callaham, D., Del Tredici, P. and Torrey, J. G. 1978. Isolation and cultivation *in vitro* of the actinomycete causing root nodulation in *Comptonia*. *Science* **199**, 899-902.

Carpenter, C. V., Robertson, L. R., Gordon, J. C. and Perry, D. A. 1984. The effect of four new *Frankia* isolates on growth and nitrogenase activity in clones of *Alnus rubra* and *Alnus sinuata*. *Can. J. For. Res.* **14**, 701-706.

Costa, A. and Rodriguez-Barrueco, C. 1976. Caracterizacion de *Streptomyces* sp. aislado de nodulos radicales de *Alnus glutinosa* (L) Gaertn. *Ann. Centro de Edafologia Biol. Aplicada.* Salamanca pp. 21-30. (Cited by D. Baker and J. G. Torrey, 1979).

Crocker, R. L. and Major, J. 1955. Soil development in relation to vegetation and surface age at Glacier Bay, Alaska. *J. Ecol.* **43**, 427-448.

Dale, M. E. 1963. Interplant alder to increase growth in strip-mine plantations. *U.S.D.A. For. Serv. Res. Nate CS-14.*pp. 4. Central States For. Expt. Stat., Columbus, Ohio.

Dalton, D. A. and Zobel, D. B. 1977. Ecological aspects of nitrogen fixation by *Purshia tridentata*. *Pl. Soil.* **48**, 57-80.

Dart, P. J. and Day, J. M. 1971. Effects of incubation temperature and oxygen tension on nitrogenase activity of legume root nodules. *Pl. Soil* **Special vol.**, 167-184.

Dawson, J. O. 1979. Nitrogen-fixing trees and shrubs. *For. Res.* 21, 8.

Dawson, J. O. 1983. Dinitrogen fixation in forest ecosystems. *Can. J. Microbiol.* 29 979-991.

Dawson, J. O. and Sun, S. H. 1981. The effect of *Frankia* isolates from *Comptonia peregrina* and *Alnus crispa* on the growth of *Alnus glutinosa, Alnus cordata* and *Alnus incana* clones. *Can. J. For. Res.* 11, 758-762.

DeBell, D. S. 1979. Future potential for the use of symbiotic nitrogen fixation in forest management. In In *Symbiotic nitrogen fixation in the management of temperate forests*, ed. Gordon, J. C., Wheeler, C. T. and Perry, D. A., pp. 451-466. School of Forestry, Oregon State University, U.S.A.

Diem, H. G. and Dommergues, Y. R. 1985. *In vitro* production of specialised reproductive torulose hyphae by *Frankia* strain ORS 021001 isolated from *Casuarina junghuhniana* root nodules. *Pl. Soil* 87, 17-29.

Diem, H. G., Gauthier, D. and Dommergues, Y. R. 1982. Isolation of *Frankia* from nodules of *Casuarina equisetifolia*. *Can. J. Microbiol.* 28, 526-530.

Diem, H. G., Gauthier, D. and Dommergues, Y. R. 1983. An effective strain of *Frankia* from *Casuarina* sp. *Can. J. Bot.* 61, 2815-2821.

Dijk, C. van. 1978. Spore formation and endophyte diversity in root nodules of *Alnus glutinosa* (L), VILL. *New Phytol.* 81, 601-615.

Dijk, C. van. 1979. Endophyte distribution in the soil. In *Symbiotic nitrogen fixation in the management of temperate forests*, ed. Gordon, J. C., Wheeler, C. T. and Perry, D. A., pp. 84-94. School of Forestry, Oregon State University, U.S.A.

Dijk, C. van. 1980. Ecological aspects of root nodule formation in *Alnus glutinosa* by *Frankia* sp. and *Penecillium nigrans*. *Acta. Bot. Neerl.* **29**, 219-220.

Dijk, C. van. 1984. Ecological aspects of spore formation in the *Frankia-Alnus* symbiosis. *Ph. D. Thesis*. University of Leiden, The Netherlands, pp. 154.

Dijk, C. van. and Merkus, E. 1976. A microscopical study of the development of a spore-like stage in the life cycle of the root-nodule endophyte of *Alnus glutinosa* (L) Gaertn. *New Phytol.* **77**, 73-91.

Dillon, J. T. and Baker, D. 1982. Variations in nitrogenase activity among pure cultured *Frankia* strains tested on actinorhizal plants as an indication of symbiotic compatibility. *New Phytol.* **92**, 215-219.

Dilworth, M. J. 1966. Acetylene reduction by nitrogen-fixing preparations from *Clostridium pasteurianum*. *Biochem. Biophys. Acta.* **127**, 285-294.

Dixon, R. O. D. 1967. Hydrogen uptake and exchange by pea root nodules. *Ann. Bot.* **31**, 179-188.

Dixon, R. O. D. 1968. Hydrogenase in pea root nodule bacteriods. *Arch. Microbiol.* **62**, 272-283.

Dixon, R. O. D. 1972. Hydrogenase in legume root nodule bacteriods: occurence and properties. *Arch. Microbiol.* **85**, 193-201.

Dixon, R. O. D. and Wheeler, C. T. 1983. Biochemical, physiological and environmental aspects of symbiotic nitrogen fixation. In *Biological Nitrogen Fixation in Forest Ecosystems: Foundations and Applications.* ed. Gordon, J. C. and Wheeler, C. T. pp. 107-171. Martinus Nijhoff / Dr. W. Junk Publishers, The Hague.

Dixon, R. O. D. and Wheeler, C. T. 1986. *Nitrogen Fixation in Plants.* Blackie, Glasgow and London.

Duncan, D.B. 1955. Multiple range and multiple F tests. *Biometrics* **11**, 1-42.

Epstein, E. 1972. *Mineral Nutrition of Plants, Principles and Perspectives.* Wiley, New York.

Ensign, J. C. 1978. Formation, properties and germination of actinomycete spores. *Ann. Rev. Microbiol.* **32**, 185-219.

Evans, G.C. 1972. The Quantitative Analysis of Plant Growth. Blackwell Scientific Publications, Oxford.

Evans, J. 1984. Silviculture of broadleaved woodland. *Forestry Commission Bull.* **62**, pp 183-187.

Fessenden, R. J. 1979. Use of actinorhizal plants for land reclamation and amenity planting in the U.S.A. and Canada. In *Symbiotic nitrogen fixation in the management of temperate forests*, ed. Gordon, J. C., Wheeler, C. T. and Perry, D. A., pp. 403-419. School of Forestry, Oregon State University, U.S.A.

Funk, D. T. 1973. Growth and development of alder plantings on Ohio strip-mine banks. In *Ecology and Reclamation of Devestated Land.* Vol. 1., ed. Hutnik, R. J. and Davis, pp.483-491. G. Gordon and Breach, New York.

Gardner, I. 1958. Nitrogen fixation in *Elaeagnus* root nodules. *Nature* **181**, 717-718.

Gardner, I. 1965. Observations on the fine structure of the endophyte of the root nodule of *Alnus glutinosa* (L) Gaertn. *Archiv. Fur Mikrobiol.* **51**, 365-383.

Gauch, H. G. 1972. *Inorganic Plant Nutrition.* Dowden, Hutchinson and Ross Inc. Stroudsburg, Pa.

Gauthier, D., Diem, H. G. and Dommergues, Y. 1981. *In vitro* nitrogen fixation by two actinomycete strains isolated from *Casuarina* nodules. *Appl. Envir. Mibrobiol.* **41**, 306-308.

Gauthier, D., Diem, H. G. and Dommergues, Y. 1985. Assessment of N_2 fixation by *Casuarina equisetifolia* inoculated with *Frankia* ORS021001 using ^{15}N methods. *Soil Biol. Biochem.* **17(3)**, 375-379.

Goh, K. M., Edmeades, D. C. and Robinson, B. W. 1978. Field measurements of symbiotic nitrogen fixation in an established pasture using acetylene reduction and a ^{15}N method. Soil Biol. Biochem. **10**, 13-20.

Granhall, U. 1982. The use of *Alnus* in energy forestry. *Proc. 2nd Natn Symp on N Fixation,* pp. 273-285. The Finnish National Fund for research and Development, Helsinki.

Gordon J. C. 1975. The productive potential of woody plants. *Iowa State J. Res.* **49**, 267-274. (Cited by J. C. Gordon and J. O. Dawson. 1979.)

Gordon, J. C. and Dawson, J. O. 1979. Potential uses of nitrogen-fixing trees and shrubs in commercial forestry. *Bot. Gaz.* **140S**, S88-S90.

Griffiths, A. P. and McCormick, L. H. 1984. Effects of soil acidity on nodulation of *Alnus glutinosa* and viability of *Frankia. Pl. Soil.* **79**, 429-434.

Hafeez, F., Akkermans, A. D. L. and Chaudhary, A. H. 1984. Morphology, physiology and infectivity of two *Frankia* isolates An1 and An2 from root nodules of *Alnus nitida. Pl. Soil* **78**, 45-59.

Hall, R. H., McNabb, C., Maynard, C. and Green, T. 1979 Toward development of optimal *Alnus glutinosa* symbioses. *Bot. Gaz.* **140S**, S120-S126.

Hallsworth, E. G., Wilson, S. B., and Adams, W. A. 1965. Effect of cobalt on the non nodulated legume. *Nature* **205**, 307.

Hardy, R. W. F., Burns, R. C. and Holsten, R. D. 1973. Applications of the acetylene-ethylene assay for measurement of nitrogen fixation. *Soil Biol. Biochem.*, 5, 47-81.

Hely, F. W. 1972. Genetic studies with wild diploid *Trifolium ambiguum* M. Bieb. with respect to time of nodulation. *Aust. J. Agric. Res.* 23, 437-446.

Hoch, G. E., Little, H. N. and Burriss, R. H. 1957. H_2 evolution from soybean root nodules. *Nature* **179**, 430-431.

Hoch, G. E. , Schneider, K. C. and Burris, R. H. 1960. Hydrogen evolution and exchange and conversion of N_2O to N_2 by soybean root nodules. *Biochem. Biophys. Acta.* 37, 273-279.

Holfeld, H. S., Mallard, C. S. and La Rue, T. A. 1979. Portable gas chromatography. *Pl. Soil*, 61, 169-188.

Hooker, J.E. and Wheeler, C.T. 1987. The effectivity of *Frankia* for nodulation and nitrogen fixation in *Alnus rubra* and *A. glutinosa*. *Physiol. Plantarum.* in press.

Houwers, A. and Akkermans, A. D. L. 1981. Influence of inoculation on yeild of *Alnus glutinosa* in The Netherlands. *Pl. Soil.* 61, 189-202.

Huss-Danell, K. and Frej, A-K. 1986. Distribution of *Frankia* in soils from forest and afforestation sites in northern Sweden. *Pl. Soil* **90**, 407-419.

Huss-Danell, K., Roelofsen, W., Akkermans, A. D. L. and Meijer, P. 1982. Carbon metabolism of *Frankia* spp. in root nodules of *Alnus glutinosa* and *Hippophae rhamnoides*. *Physiol. Plant.* **54**, 461-466.

Huss-Danell, K. and Sellstedt, A. 1985. Nitrogenase activity in response to darkening and defoliation of *Alnus incana*. *J. Exp. Bot.* **36**, 1352-1358.

Jalal, M.A.F. and Read, D.J. 1983. The organic composition of *Calluna* heathland soil with special reference to phyto- and fungitoxicity. I. isolation and identification of organic acids. *Pl. Soil* **70**, 257-272.

Jensen, V. 1968. The plate count technique. In *The Ecology of Soil Bacteria*. ed. Gray, T. and Parkinson, D. pp. 158-170. Liverpool University Press, Liverpool.

Jobidon, R. and Thibault, J. R. 1982. Allelopathic growth inhibition of nodulated and unnodulated *Alnus crispa* seedlings by *Populus balsamifera*. *Amer. J. Bot.* **69**, 1213-1223.

Jobling, J. and Stevens, F. R. W. 1980. Establishment of trees on regraded colliery and spoil heaps. *Forestry Commission Occasional* Paper 7. Forestry Commission, Edinburgh (Cited by J. Evans, 1984).

Kappel, M. and Wartenberg, H. 1958. Der Formenwechsel des *Actinomycetes alni* Peklo in den Wurzeln von *Alnus glutinosa* Gaertn. *Arch. Mikrobiol.* **30**, 46. (Cited in Dijk van and Merkus, 1976).

Kliewer, M. and Evans, H. J. 1962. B_{12} coenzyme content of the nodules from legumes, alder and of *Rhizobium meliloti*. *Nature* **194**, 108.

Knowlton, S., Berry, A. and Torrey, J. G. 1980. Evidence that associated soil bacteria may influence root hair infection of actinorhizal plants by *Frankia*. *Can. J. Microbiol.* **26**, 971-977.

Knowlton, S. and Dalton, J. O. 1982. Interaction of substrate pH and *Pseudomonas cepacia* in promoting *Frankia* nodulation of *Alnus crispa*. *Plant Physiol.* **69**, (4 suppl.), 109.

Kohl, D. H., Bryan, B. A., Shearer, G. and Virginia, R. 1981. Concerning the heterogeneity of the natural abundance of ^{15}N in soil nitrogen. *Soil Sci. Soc. Am. J.* **45**, 450-451.

Kohl, D. H. and Shearer, G. 1980. Isotopic fractionation associated with symbiotic N_2 fixation and uptake of NO_3^- by plants. *Plant Physiol.* **66**, 51-56.

Lalonde, M. 1978. Confirmation of the infectivity of a free-living actinomycete isolated from *Comptonia peregrina* root nodules by immunological and ultrastructural studies. *Can. J. Bot.* **56**, 2621-2635.

Lalonde, M. 1979. Immunological and ultrstructural demonstration of nodulation of the European *Alnus glutinosa* (L) Gaertn. host plant by an actinomycetal isolate from the North American *Comptonia peregrina* (L) Coult. root nodule. *Bot. Gaz.* **140S**, S35-S43.

Lalonde, M. and Calvert, H. E. 1979. Production of *Frankia* hyphae and spores as an infective inoculant for *Alnus* species. In *Symbiotic nitrogen fixation in the management of temperate forests*, ed. Gordon, J. C., Wheeler, C. T. and Perry, D. A., pp.95-110. School of Forestry, Oregon State University, U.S.A.

Lalonde, M., Calvert, H. E. and Pine, S. 1981. Isolation and use of *Frankia* strains in actinorhizal formation. In *Current perspective in nitrogen fixation*, ed. Gibson, A. H. and Newton, W. E., pp. 296-299. Australian Academy of Science, Canberra.

Lalonde, M., Knowles, R. and Fortin, J. A. 1975. Demonstration of the isolation of a non-infective *Alnus crispa* v. mollis fern nodule endophyte by morphological, immunolabelling and whole cell composition studies. *Can. J. Microbiol.* **21**, 1901-1920.

Lancelle, S. A., Torrey, J. G., Hepler, P. K. and Callaham, D. A. 1985. Ultrastructure of freeze- substituted *Frankia* strain HFPCcI3, the actinomycete isolated from root nodules of *Casuarina cunninghamiana*. *Protoplasma* **127**, 64-72.

Lawrie, A. C. 1982. Field nodulation in nine species of *Casuarina* in Victoria. *Aust. J. Bot.* **30**, 447-460.

Lechevalier, M. P. 1983. Cataloging *Frankia* strains. *Can. J. Bot.* **61**, 2964-2967.

Lechevalier, M. P., Baker, D. and Horriere, F. 1983. Physiology, chemistry, serology, and infectivity of two *Frankia* isolates from *Alnus incana* subsp. *rugosa*. *Can. J. Bot.* **61**, 2826-2834.

Lechevalier, M. P. and Lechevalier, H. 1979. The taxanomic position of the endophytes. In *Symbiotic nitrogen fixation in the management of temperate forests*, ed. Gordon, J. C., Wheeler, C. T. and Perry, D. A., pp.111-122. School of Forestry, Oregon State University, U.S.A.

Lechevalier, M. P. and Ruan, J. 1984. Physiology and chamical diversity of *Frankia* spp. isolated from nodules of *Comptonia peregrina* (L) Coult. and *Ceanothus americanus* (L). *Pl. Soil* **78**, 15-22.

Lie, T. A., Akkermans, A. D. L. and Egeraat van, A. W. S. M. 1984. Natural variation in symbiotic nitrogen-fixing *Rhizobium* and *Frankia* spp. *Antonie van Leeuwenhoek* **50**, 489-503

Lines, R. and Brown , J. R. 1982. Broadleaves for the uplands. In *Broadleaves in Britain: future management and research*, ed. Malcolm, D. C., Evans, J. and Edwards, P. N., pp. 141-149. Institute of Chartered Foresters, Edinburgh. (Cited by J. Evans, 1984).

Lopez, M. F. and Torrey, J. G. 1985. Enzymes of glucose metabolism in *Frankia* sp. *J. Bact.* **162**, 110-116.

Lopez, M. F., Whaling, C. S. and Torrey, J. G. 1983 The polar lipids and free sugars of *Frankia* in culture. *Can. J. Bot.* **61**, 2834-2842.

McNeil, R. E. and Carpenter, P. L. 1979. The effect of temperature, solar radiation and moisture on the reduction of acetylene by excised root nodules from *Alnus glutinosa*. *New Phytol.* **82**, 459-465.

McVean, D. N. 1956. Ecology of *Alnus glutinosa* (L) Gaertn. V. Notes on some British alder populations. *J. Ecol.* **44**, 321-330.

McVean, D. N. 1962. Botanical Research. Scots Pine, Alder and Bog myrtle. *Report Nature Conservancy, London.*

Malcolm, D.C., Hooker, J. E. and Wheeler, C.T. 1985. Frankia symbiosis as a source of nitrogen in forestry: a case study of symbiotic nitrogen-fixation in a mixed *Alnus-Picea* plantation in Scotland. *Proc. Roy. Soc. Edin.* **85B**, 263-282.

Maynard, C. A. 1980. Host-symbiont interactions between *Frankia* strains and *Alnus* open-pollinated families. *Ph.D. Thesis*, Iowa State University.

Meesters, T. M., Genesen van, S. Th. and Akkermans, A. D. L. 1985. Growth, acetylene reduction activity and localization of nitrogenase in relation to vesicle formation in *Frankia* strains Cc1.17 and Cp1.2. *Arch. Microbiol.* **143**, 137-142.

Mengel, K. and Kirby, E. A. 1978. *Principles of Plant Nutrition.* International Potash Institute, Berne, Switzerland.

Miguel, C., Canizo, A., Costa, A. and Rodriguez-Barrueco, C. 1978. Some aspects of the *Alnus*-type root nodule symbiosis. In *Limitations and Potentials for Biological Nitrogen Fixation in the Tropics*. ed. Dobereiner, J. and Burris, R. H. pp. 121-133,Plenum Press.

Minchin, F. R., Witty, J. F., Sheehy, J. E. and Muller, M. 1983. A major error in the acetylene reduction assay: decreases in nodular nitrogenase activity under assay conditions. *J. Exp. Bot.* **34**, 641-649.

Mitchell, R. L. 1972. Cobalt in soil and its uptake by plants. *Agrochemica* **16**, 521-532.

Moiroud, A. and Faure-Raynaud, M. 1983. Effects of some herbicides on the growth and infectivity of *Frankia* strains. *Pl. Soil* **74**, 133-136.

Moiroud, A., Simonet, P. and Faure-Raynaud, M. 1985. Influence of four herbicides and one fungicide on *Frankia* strains grown *in vitro*, on nodule formation and on Alder seedlings growth. *Ann. Sci. For.* **42**, 339-350.

Moore, A. W. 1964. Note on non-leguminous nitrogen fixing plants in Alberta. *Can. J. Bot.* **42**, 952-955.

Murdoch, J. and Barnes, J.A. 1974. *Statistical Tables*. Macmillan, London and Cambridge. pp. 46.

Murray, M. A., Fontaine, M. S. and Tjepkema, J. D. 1984. Oxygen protection of nitrogenase in *Frankia* sp. HFPArI3. *Arch. Microbiol.* **139**, 162-166.

Nesme, X, Normand, P., Tremblay, F. M. and Lalonde, M. 1985. Nodulation speed of *Frankia* sp. on *Alnus glutinosa, Alnus crispa* and *Myrica gale*. *Can. J. Bot.* **63**, 1292-1295.

Newton, M.,El Hassan, B. A. and Zavitkovski, J. 1968. Role of red alder in Western Oregon forest succession. In *Proc. Symp. 'Biology of Alder'*. ed. Trappe, J. M., Franklin, J. F., Tarrant, R. F. and Hansen G. M., pp. 73-84. Pacific N. W. For. and Range Exp. Sta., U.S.D.A., Portland, Oregon.

Noel, A. R. A. 1964. A staining and mounting combination for sections of plant tissues. *Stain Technol.* **39**, 324.

Normand, P. and Lalonde, M. 1982. Evaluation of *Frankia* strains isolated from provenances of two *Alnus* species. *Can. J. Microbiol.* **28**, 1133-1142.

Normand, P., Simonet, P. Butour, J. L., Rosenberg, C., Moiroud, A. and Lalonde, M. 1983. Plasmids in *Frankia*. *J. Bacteriology* **155**, 32-35.

Peklo, J. 1910. Die pflanzlichen Aktinomykosen Centralbl. *Bakteriol. Parasit.* II. **27**, 451-579. (Cited by D. Baker and J. G. Torrey, 1979).

Perinet, P., Brouillette, J. G., Fortin, J. A. and Lalonde, M. 1985. Large scale inoculation of actinorhizal plants with *Frankia*. *Pl. Soil* **87**, 175-183.

Perradin, Y., Mottet, M. J. and Lalonde, M. 1983. Influence of phenolics on *in vitro* growth of *Frankia* strains. *Can. J. Bot.* **61**, 2807-2814.

Phelps, A. S. and Wilson, P. W. 1941. Occurrence of hydrogenase in nitrogen-fixing organisms. *Proc. Soc. Exp. Biol. and Med.* **47**, 473-476.

Pommer, E. 1956. Beitrage zur anatomie und biologie der wurzelknollchen von *Alnus glutinosa* Gaertn. *Flora* **143**, 603-634. (Cited by D. Baker and J. G. Torrey, 1979).

Pommer, E. 1959. Uber die isolierung des endophyten aus den wurzelknollchen *Alnus glutinosa* Gaertn. und uber erfolgreiche reinfektionsversuche. *Ber. Dt. Bot. Ges.* **72**, 138-150.

Postgate, J. R. 1981. *The Fundamentals of Nitrogen Fixation*. Cambridge University Press, Cambridge.

Quispel, A. 1954. Symbiotic nitrogen fixation in non-leguminous plants. II. The influence of the inoculation density and external factors on the nodulation of *Alnus glutinosa* and its importance to our understanding of the mechanism of infection. *Acta. Bot. Neerl.* 3, 512-532.

Quispel, A. 1958. Symbiotic nitrogen fixation in non-leguminous plants. IV. The influence of some environmental conditions on different phases of the nodulation process in *Alnus glutinosa*. *Acta. Bot. Neerl.* 7, 191-204.

Quispel, A. 1960. Symbiotic nitrogen fixation in non-leguminous plants. V. The growth requirements of the endophyte of *Alnus glutinosa*. *Acta. Bot. Neerl.* 9, 380-396.

Quispel, A., Burggraaf, A. J. P., Borsje, H. and Tak, T. 1983. The role of lipids in the growth of *Frankia* isolates. *Can. J. Bot.* 61, 2801-2806.

Quispel, A. and Tak, T. 1978. Studies on the growth of the endophyte of *Alnus glutinosa* (L) VILL. in nutrient solutions. *New Phytol.* 81, 587-600.

Radwan, M. A., Harrington, C. A. and Kraft, J. M. 1984. Litterfall and nutrient returns in red alder stands in western Washington. *Pl. Soil* 79, 343-351.

Read, D.J. 1986. Non-nutritional effects of mycorrhizal infection. In *Physiological and Genetical Aspects of Mycorrhizae. Proceedings of the 1st European Symposium on Mycorrhizae Dijon 1-5 July 1985.* ed Gianinazzi-Pearson, V. and Gianinazzi, S. pp. 169-176. INRA, Paris.

Reddell, P. and Bowen, G. D. 1985. Do single nodules of Casuarinaceae contain more than one *Frankia* strain? *Pl. Soil* 88, 275-279.

Red'ko, G. I. 1958. Influence of the black alder (*Alnus glutinosa* Gaertn.) on the productivity of the Canadian poplar (*Populus canadensis* Mich.). *TT66-51093. U.S. Dept. Comm.* , Clearinghouse for Fed. Sci. Tech. Inf., Springfeild, VA 22151. (Cited by J. Zavitkovski, E.A. Hansen and H. A. McNeel, 1979).

Ridgman, W.J. 1975. *Experimentation in Biology. An Introduction to Design and Analysis.* Blackie, Glasgow and London. pp. 233.

Rodriquez-Barrueco, C. 1968. The occurrence of the root-nodule endophyte of *Alnus glutinosa* and *Myrica gale* in soils. *J. Gen. Microbiol.* 52, 189-194.

Roderiguez-Barrueco, C. and Bond, G. 1968. Nodule endophytes in the genus *Alnus.* In *Biology of Alder.* ed. Trappe, J. M., Franklin, J. F., Tarrant, R. F. and Hansen G. M., pp. 185-192. Pacific N. W. For. and Range Exp. Sta., U.S.D.A., Portland, Oregon.

Roderiguez-Barrueco, C. and Bond, G. 1976. A discussion of the results of cross-inoculation trials between *Alnus glutinosa* and *Myrica gale.* In *Symbiotic Nitrogen Fixation in Plants.* Vol. 7. pp. 561-565. International Biological Programme. Cambridge University Press, Cambridge.

Rodriquez-Barrueco, C., MacKintosh, A. H. and Bond, G. 1970. Some effects of combined nitrogen on the nodule symbiosis of *Casuarina* and *Ceanothus. Pl. Soil* 33, 129-139.

Roelofsen, W. and Akkermans, A. D. L. 1979. Uptake and evolution of H_2 and reduction of C_2H_2 by root nodules and nodule homogenates of *Alnus glutinosa*. *Pl. Soil* 52, 571-578.

Schachtschabel, P. 1963. Optimum pH, phosphorus and potassium in arable soils. *Landw. Forsch.* 17, Sonderheft, 60-82. (cited by Mengel and Kirby 1978).

Schaede, R. 1933. Uber die Symbionten in den Knollchen der Erle und des Sanddornes und die cytologischen Verhaltnisse in ihnen. *Planta (Berl.)* 19, 389. (Cited in Dijk Van and Merkus, 1976).

Schubert, K. R. and Evans, H. J. 1976. Hydrogen evolution: a major factor affecting the efficiency of nitrogen fixation in nodulated symbionts. *Proc. Nat. Acad. Sci. USA.* 73, 1207-1211.

Schubert, K. R. and Evans, H. J. 1977. The relation of hydrogen reactions to nitrogen fixation in nodulated symbionts. In *Recent Developments in Nitrogen Fixation.* ed. Newton, W. E., Postgate, J. R. and Rodriquez-Barrueco, Cambridge Academic Press, London.

Sellstedt, A., Huss-Danell, K and Ahlqvist, A. S. 1986. Nitrogen fixation and biomass production in symbioses between *Alnus incana* and *Frankia* strains with different hydrogen metabolism. *Physiol. Plant.* 66, 99-107.

Shipton, W. A. and Burggraaf, A. J. P. 1982. A comparison of the requirements for various carbon and nitrogen sources and vitamins in some *Frankia* isolates. *Pl. Soil* 69, 149-161.

Shipton, W. A. and Burggraaf, A. J. P. 1983. Aspects of the cultural behaviour of *Frankia* and possible ecological implications. *Can. J. Bot.* 61, 2783-2792.

Silver, W. S. and Mague, T. 1970. Assessment of nitrogen fixation in terrestrial environments in field conditions. *Nature* (London) 227, 378-379.

Silvester, W. B. 1977. Dinitrogen fixation by plant associations excluding legumes. In *A Treatise on Dinitrogen Fixation*. ed. Hardy, R. W. F. and Gibson, A. H., pp. 141-190. Wiley, New York.

Silvester, W. B. 1983. Analysis of nitrogen fixation. In *Biological Nitrogen Fixation in Forest Ecosyatems: Foundations and Applications*. ed. Gordon, J. C. and Wheeler, C. T., Martinus Nijhoff / Dr. W. Junk Publishers.

Simon, L., Stein, A., Cote, S. and Lalonde, M. 1985. Performance of *in vitro* propagated *Alnus glutinosa* (L) Gaertn. clones inoculated with *Frankiae*. *Pl. Soil*. 87, 125-133.

Simonet, P., Normand, P., Moiroud, A. and Lalonde, M. 1985. Restriction enzyme digestion patterns of *Frankia* plasmids. *Pl. Soil* 87, 49-60.

Sprent, J. I. and Bradford, A. M. 1977. Nitrogen fixation in field beans *Vicia faba* as affected by population density, shading and its relationship with soil moisture. *J. Agric. Sci.* 88, 303-310.

Stevenson, F. J. 1967. Organic acids in soils. In *Soil Biochemistry*, ed. McLaren, A. D. and Peterson, G. H. pp. 119-146. Marcel Dekker Inc., New York.

Stewart, W. D. P. 1963. *Z. Allgem. Mikrobiol.* 3, 152-156 (cited in Bond, 1967).

Szalay, A. and Szilagyi, M. 1968. Laboratory experiments on the retention of micronutrients by peat humic acids. *Pl. Soil.* 29, 219-224.

Tarrant, R. F. and Miller, R. I. 1963. Accumulation of organic matter and soil nitrogen beneath a plantation of red alder and Douglas-fir. *Soil Sci. Soc. Am. Proc.* 27, 231-234.

Tarrant, R. F. and Trappe, J. M. 1971. The role of *Alnus* in improving the forest environment. *Pl. Soil* (Special volume), 335-348.

Tessier, Du., Cross, E., Jung, G. and Bariteau, M. 1984. Alder-*Frankia* interaction and alder-popular association for biomass production. *Pl. Soil* 78, 235-243.

Tisa, L., McBride, M. and Ensijn, J. C. 1983. Studies of growth and morphology of *Frankia* strains EAN1pec, EUI1c, CpI1 and ACN1^{AG}. *Can. J. Bot.* 61, 2768-2773.

Tjepkema, J. D. Ormerod, W., and Torrey, J. G. 1980. Vesicle formation and acetylene reduction activity in *Frankia* sp. CPI1 cultured in defined nutrient media. *Nature* (London) 287, 633-635.

Tjepkema, J. D. Ormerod, W., and Torrey, J. G. 1981. Factors affecting vesicle formation and acetylene reduction (nitrogenase activity) in *Frankia* sp. CPI1. *Can. J. Bot.* **27**, 815-823.

Tjepkema, J. D., Schwintzer, C. R. and Benson, D. R. 1986. Physiology of actinorhizal nodules. *Ann. Rev. Plant Physiol. 209-232.*

Torrey, J. G. 1983. Root development and root nodulation in Casuarina. In *Casuarina Ecology, Management and Utilisation.* ed. Midgley, S. J., Turnbull, J. W. and Johnston, R. D., pp. 180-192. CSIRO, Melbourne, Australia.

Torrey, J. G. 1983. *Casuarina*: Actinorhizal di-nitrogen-fixing tree of the tropics. In *Casuarina Ecology, Management and Utilisation.* ed. Midgley, S. J., Turnbull, J. W. and Johnston, R. D., pp. 180-192. CSIRO, Melbourne, Australia.

Torrey, J. G. 1987. Endophyte sporulation in actinorhizal nodules. *Physiol. Plant.* **in press.**

Torrey, J. G. and Callaham, D. 1982. Structural features of the vesicle of *Frankia* sp. CPI1 in culture. *Can. J. microbiol.* **28**, 749-757.

Tyson, J. H. and Silver, W. S. 1979. Relationship of ultrastructure to acetylene reduction (N_2 fixation) in root nodules of *Casuarina. Bot. Gaz.* **140**, 544-548.

VandenBosch, K. A. and Torrey, J. G. 1984. Consequences of sporangial development for nodule function in root nodules of *Comptonia peregrina* and *Myrica gale*. *Plant Physiol.* **76**, 556-560.

VandenBosch, K. A. and Torrey, J. G. 1985. Development of endophytic *Frankia* sporangia in field - and laboratory - grown nodules of *Comptonia peregrina* and *Myrica gale*. *Amer. J. Bot.* **72**, 99-108.

Vemura, S. 1961. Studies on the root nodules of alders (*Alnus* spp.). VI. About the morphological and physiological properties of *Streptomycetes* usually isolated from alder and some non-leguminous root nodules (*Myrica gale, Elaegnus umbellata* and *Casuarina equisetifolia*). *Sci. Rep. Agr. For. Fish. Res. Council* (Tokyo) **7**, 1-86. (Cited by D. Baker and J. G. Torrey, 1979).

Visser, S. A. 1985. Physiological action of humic substances on microbial cells. *Soil Biol. Biochem.* **17**, 457-462.

Wada, E., Kadonoga, T. and Matsuo, S. 1975. ^{15}N abundance in nitrogen of naturally occurring substances and assessment of denitrification from a global viewpoint. *Geochem. J.* **9**, 139-148.

Wheeler, C. T. 1984. *Frankia* and its symbiosis in non-legume (actinorhizal) root nodules. In *Current Developments in Biological Nitrogen Fixation*. ed. Subba Rao, N. S., pp. 173-195. Edward Arnold (Publishers) Ltd., London.

Wheeler, C. T. and Bowes, B. G. 1974. Effects of light and darkness on nitrogen fixation by root nodules of *Alnus glutinosa* in relation to their cytology. *Z. fur Pflanzenphysiol.* 71, 71-75.

Wheeler, C. T., Cameron, E. M. and Gordon, J. C. 1978. Effects of handling and surgical treatments on nitrogenase activity in root nodules of *Alnus glutinosa*, with special reference to the application of indole-acetic acid. *New Phytol.* 80, 175-178.

Wheeler, C.T., Crozier, A. and Sandberg, G. 1984. The biosynthesis of indole-3-acetic acid by *Frankia*. *Pl. Soil* 78, 99-104.

Wheeler, C. T., Hooker, J. E., Crowe, A. and Berrie, A. M. M. 1986. The improvement and utilisation in forestry of nitrogen fixation by actinorhizal plants with special reference to *Alnus* in Scotland. *Pl. Soil* 90, 393-406.

Wheeler, C. T. and McLaughlin, M. E. 1979. Environmental nodulation of nitrogen fixation in actinomycete nodulated plants. In Symbiotic nitrogen fixation in the management of temperate forests, ed. Gordon, J. C., Wheeler, C. T. and Perry, D. A., pp.124-143. School of Forestry, Oregon State University, U.S.A.

Wheeler, C. T., McLaughlin, M. E. and Steele, P. 1981. A comparison of symbiotic nitrogen fixation in Scotland in *Alnus glutinosa* and *Alnus rubra*. *Pl. Soil* 61, 169-188.

Wilson, P. W., Umbreit, W. W. and Lee, S. B. 1938. Mechanism of symbiotic nitrogen fixation. IV. Specific inhibition by hydrogen. Biochem. J. 32, 2084. (Cited by Evans *et al.* 1984).

Wollum, A. G. II, Youngberg, C. T. and Gilmour, C. M. 1966. Characterisation of a *Streptomyces* sp. isolated from root nodules of *Ceanothus velutinus*. Dougl. *Proc. Soil Sci. Soc. Am.* 30, 463-467.

Yates, M. G. and Eady, R. R. 1980. Physiology and regulation of nitrogen fixation. In *Recent Advances in Biological Nitrogen Fixation.* ed. Subba-Rao, N. S. , pp. 88-120. Arnold, London.

Youngberg, C. T.and Wollum, A. G. 1976. Nitrogen accretion in developing *Ceanothus velutinus* soils. *Soil Sci. Soc. Amer. J.* 40, 109-112.

Zavitkovski, J., Hansen, E. A. and McNeel, H. A. 1979. Nitrogen-fixing species in short rotation systems for fiber and energy production. *In Symbiotic nitrogen fixation in the management of temperate forests*, ed. Gordon, J. C., Wheeler, C. T. and Perry, D. A., pp.388-402. School of Forestry, Oregon State University, U.S.A.

Zeigler, H. and Huser, R. 1963. *Naturek* 199, 508. (Cited by G. Bond, 1967).

Zhang, Z., Lopez, M. F. and Torrey, J. G. 1984. A comparison of cultural characteristics and infectivity of *Frankia* isolates from root nodules of *Casuarina* species. *Pl. Soil* 78, 79-90.

Published papers resulting in whole or in part from the work of this Thesis.

Hooker, J. E. and Wheeler, C.T. 1987. The effectivity of *Frankia* for nodulation and nitrogen fixation in *Alnus rubra* and *A. glutinosa*. *Physiol. Plantarum in press.*

Malcolm, D.C., Hooker, J.E. and Wheeler, C.T. 1985. Frankia symbiosis as a source of nitrogen in forestry: a case study of symbiotic nitrogen-fixation in a mixed *Alnus-Picea* plantation in Scotland. *Proc. Roy. Soc. Edin.* **85B**, 263-282.

Wheeler, C.T., Hooker, J.E., Crowe, A. and Berrie, A.M.M. 1986. The improvement and utilisation in forestry of nitrogen fixation by actinorhizal plants with special reference to *Alnus* in Scotland. *Pl. Soil*, **90**, 393-406.