Production of indole-3-acetic acid by *Rhizobium phaseoli* 8002 in relation to nodulation of *Phaseolus vulgaris* L.

By

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This thesis is dedicated to Mum and Dad with love and thanks.

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Declaration

I hereby declare that the work presented in the following thesis is my own except where otherwise acknowledged, and that the thesis is of my own composition. No part of this work has been previously presented for any other degree.

Signed

Janice A. Smith

September, 1992

Summary

The research reported in this thesis was concerned with the production of indolic compounds, particularly indole-3-acetic acid (IAA) by the nitrogen-fixing bacterium *Rhizobium phaseoli* strain 8002 and its leguminous host, *Phaseolus vulgaris*. These organisms are involved in a highly complex symbiosis in which bacterial IAA production has been postulated as playing an important physiological role, either in the early stages of infection or later during the development and maintenance of the mature nodule. Experiments were designed in particular to determine whether **(a)** differences in pathways of IAA metabolism between bacterium and host plant might be used as 'markers' for IAA production by the bacterium in the symbiotic state and **(b)** whether there is a correlation between IAA production by *Rhizobium* and the ability to nodulate the leguminous host plant.

The first part of this project examined production of indoles by *Rhizobium phaseoli* in culture in tryptophan-supplemented and minus media. The spectrum of indoles detected was identical in both (+) and (-) media, with indole acetic acid (IAA), indole lactic acid (ILA), indole ethanol (IEt), indole aldehyde (IAld) and indole methanol (IM) being identified by reversed-phase and normal phase HPLC. MS analysis confirmed the presence of IAA, ILA, IEt and IAld. The tryptophan-supplemented cultures however, showed a large increase in accumulation, particularly of IAA, IEt and ILA.

Labelled metabolic feeds of [^{3}H]-tryptophan, the precursor of IAA, to *Rhizobium* in culture, resulted in the production of labelled IAA, ILA, IEt, IAld and IM, while feeding with [^{14}C]-IAA, led to production of labelled IEt, IM and IAld.

Preliminary results investigating synergism between IAA and gibberellic acid (GA_3), both of which are synthesised by the bacterium, indicated an increase in production at low GA_3 concentrations but this effect was not reproducible in subsequent experiments.

The second part of the research focused on the feeding of radiolabelled IAA and tryptophan to detached root and nodule segments of *Phaseolus vulgaris* in order to compare the spectrum of indolic compounds produced with the bacterium. The addition of radiolabelled tryptophan gave rise to labelled IAA, ILA, IEt, IAld and IM in nodule tissue, while in roots no indolic compounds were detected. The addition of [^{14}C]-IAA resulted in the production of labelled IM and IAld in both root and nodule tissue. The spectrum of metabolic products in roots and nodules was therefore identical to those produced by the bacterium *in vitro* and hence it was impossible to discriminate between bacterial and host IAA production in the nodule.

From the identification of indoles secreted by *R. phaseoli* and the analysis of metabolism of radiolabelled tryptophan and IAA, it was postulated that the pathway of IAA synthesis from tryptophan is via IPyA and IAAld, and additionally that IAA is catabolised by sequential decarboxylative oxidation, involving loss of the acidic side chain, to IAld and IM.

The final part of the project involved the isolation of *R. phaseoli* mutants with altered levels of IAA accumulation using transposon mutagenesis. A number of colonies with increased or decreased IAA accumulation in the medium, significantly different from the wild-type, were isolated. Hybridisation showed that transposon DNA was present only in the mutant DNA and that transposition, not plasmid recombination, was responsible for the mutations observed.

Differences between the lowest and the highest IAA-producers and the wild-type were of the order of 4.3-fold higher and 100-fold lower per litre of culture medium. Analysis with time of the mutants with the highest and lowest IAA accumulation revealed that the differences were significant at all stages of culture growth. These strains were inoculated onto *Phaseolus vulgaris* but no significant differences were found for nitrogen fixation and dry weight between plants inoculated with wild-type and mutant bacteria. The wild-

type however, was apparently more infective than the mutant strains. Re-isolation of the cells from the nodules revealed that only a residual resistance to neomycin remained in the population and additionally, there were no significant differences between re-isolated wild-type and mutant (neomycin resistant and sensitive mixture) bacteria in IAA accumulation. It appeared therefore that the Tn5 had been lost from the mutant cells through multiplication in the rhizosphere or inside the root, where there was obviously no antibiotic selective pressure. This may have resulted in the loss of the transposon and the reversion of the mutant to the wild-type.

The foundations have been laid for further work on *Rhizobium* mutants with altered IAA levels. It is clear however that a mutation stable from generation to generation, created through either chemical mutagenesis or a transposon with improved stability, will be required for future mutant studies *in planta.*

Abbreviations

The S.I. system of units has been used throughout this thesis. Other abbreviations are listed below:

cv.	cultivar
dpm	disintegrations per minute
E. coli	*Escherichia coli*
GA_3	gibberellic acid
GC-MS	gas chromatography-mass spectrometry
HPLC	high performance liquid chromatography
IAA	indole-3-acetic acid
IAAld	indole-3-acetaldehyde
IAAm	indole-3-acetamide
IAAsp	indole-3-acetylaspartic acid
IAld	indole-3-aldehyde
IAN	indole-3-acetonitrile
IAOx	indole-3-acetaldoxime
IBA	indole-3-butyric acid
ICA	indole-3-carboxylic acid
IEt	indole-3-ethanol
IGA	indole-3-glycolic acid
IGox A	indole-3-glyoxylic acid
ILA	indole-3-lactic acid
IM	indole-3-methanol
IPA	indole-3-propionic acid
IPyA	indole-3-pyruvic acid
MO	3-methyloxindole
m^+	molecular ion
m/z	mass/charge ratio
Nm	neomycin
OxIAA	oxindole-3-acetic acid
OxIM	oxindole-3-methanol
PVP	polyvinylpyrrolidone
RP	reversed-phase
r.t.	room temperature
S.E.	standard error of the mean
TLC	thin-layer chromatography
Tn	transposon

Statistical analyses

Statistical analyses of data were carried out using the statistical package STATGRAF.

The mean and its standard error formulas were:

$$\bar{X} = \frac{\Sigma x}{n} \quad ; \text{and} \qquad SE = \sqrt{\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n(n-1)}}$$

Where: n = number of values of x, Σx = sum of values, $\bar{X}$ = the mean of values, Σx^2 = sum of squares of values, $(\Sigma x)^2$ = square of the sum of values and SE = the standard error of the mean.

When comparing two samples for statistical significance the *t*-test method was used to determine the difference between their means and the value of *t*.

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{(S_1)^2}{n_1} + \frac{(S_2)^2}{n_2}}}$$

Where: $(S_1)^2 = \dfrac{\Sigma(x_1)^2 - \frac{(\Sigma x_1)^2}{n_1}}{n_1 - 1}$; $(S_2)^2 = \dfrac{\Sigma(x_2)^2 - \frac{(\Sigma x_2)^2}{n_2}}{n_2 - 1}$

Where: $\bar{X}_1$ and $\bar{X}_2$ = the means of samples 1 and 2, n_1 and n_2 = the number of values of samples 1 and 2, and $(S_1)^2$ and $(S_2)^2$ = the variances of samples 1 and 2, respectively.

CHAPTER 1

INTRODUCTION

1.1. Indole-3-acetic acid and related compounds

1.1.1. General introduction

Indole-3-acetic acid (IAA, **1**) is a growth regulator produced by higher and lower plants and by certain fungi and bacteria. The indole (**2**) molecule is systematically, 1 *H*-benzo(b)pyrrole; the nitrogen atom is commonly designated position 1, the carbon atoms of the bicyclic ring being numbered consecutively anticlockwise with the ring junction carbons being 3*a* and 7*a*. In IAA, the side-chain carbons are usually labelled 1' and 2' (**Fig. 1.1**).

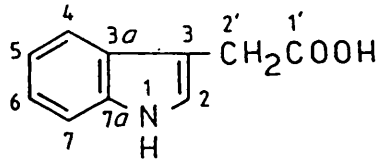
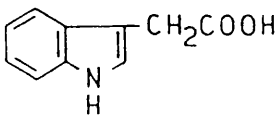


Fig. 1.1. Numbering system of the indole ring and IAA (Reproduced from Sandberg *et al.*, 1987).

In addition to IAA, there are a number of other naturally occuring indolic compounds. The majority of these are believed to be biosynthetic intermediates, catabolites or conjugated forms of IAA and some are themselves active growth substances, for example, indole-3-butyric acid (IBA, **3**) and indole-3-propionic acid (IPA, **4**) (Badenoch-Jones *et al.*, 1984).

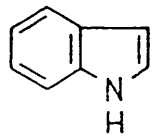
1.1.2. Biosynthesis of IAA

It is generally accepted that the biosynthetic precursor of IAA is the aromatic amino acid L-tryptophan (**5**) (Cohen and Bialek, 1984) which, in common with many other compounds, is produced via the shikimic acid pathway (**Figure 1.2.**). Beyond tryptophan the pathway to IAA can follow a number of different routes. Although the exact pathways of IAA biosynthesis operating *in vivo* in higher plants are uncertain (Cohen and



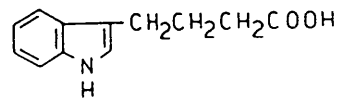
indole-3-acetic acid

1



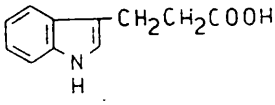
indole

2



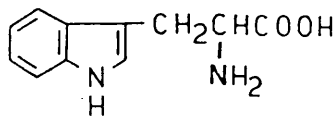
indole-3-butyric acid

3



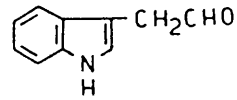
indole-3-propionic acid

4



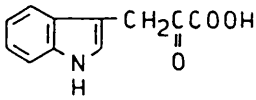
L-tryptophan

5



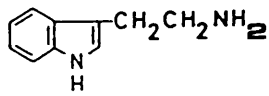
indole-3-acetaldehyde

6



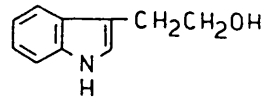
indole-3-pyruvic acid

7



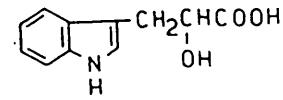
tryptamine

8



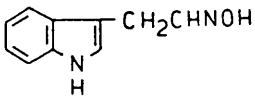
indole-3-ethanol

9



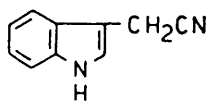
indole-3-lactic acid

10



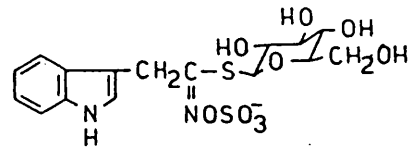
indole-3-acetaldoxime

11



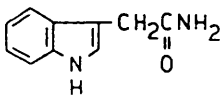
indole-3-acetonitrile

12



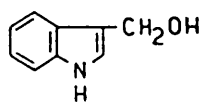
indole-3-methylglucosinolate

13



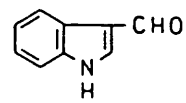
indole-3-acetamide

14



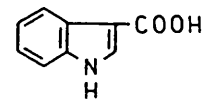
indole-3-methanol

15



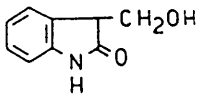
indole-3-aldehyde

16



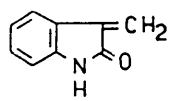
indole-3-carboxylic acid

17



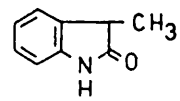
oxindole-3-methanol

18



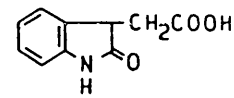
3-methyleneoxindole

19



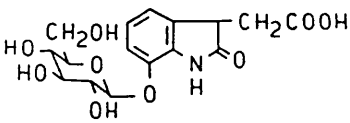
3-methyloxindole

20



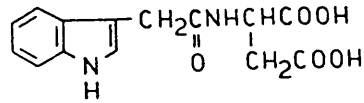
oxindole-3-acetic acid

21



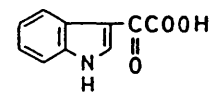
7-(*O*-β-glucosyl)-oxindole-3-acetic acid

22



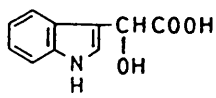
indole-3-acetylaspartic acid

23



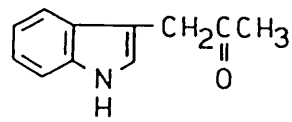
indole-3-glyoxylic acid

24



indole-3-glycolic acid

25



indole-3-acetone

26

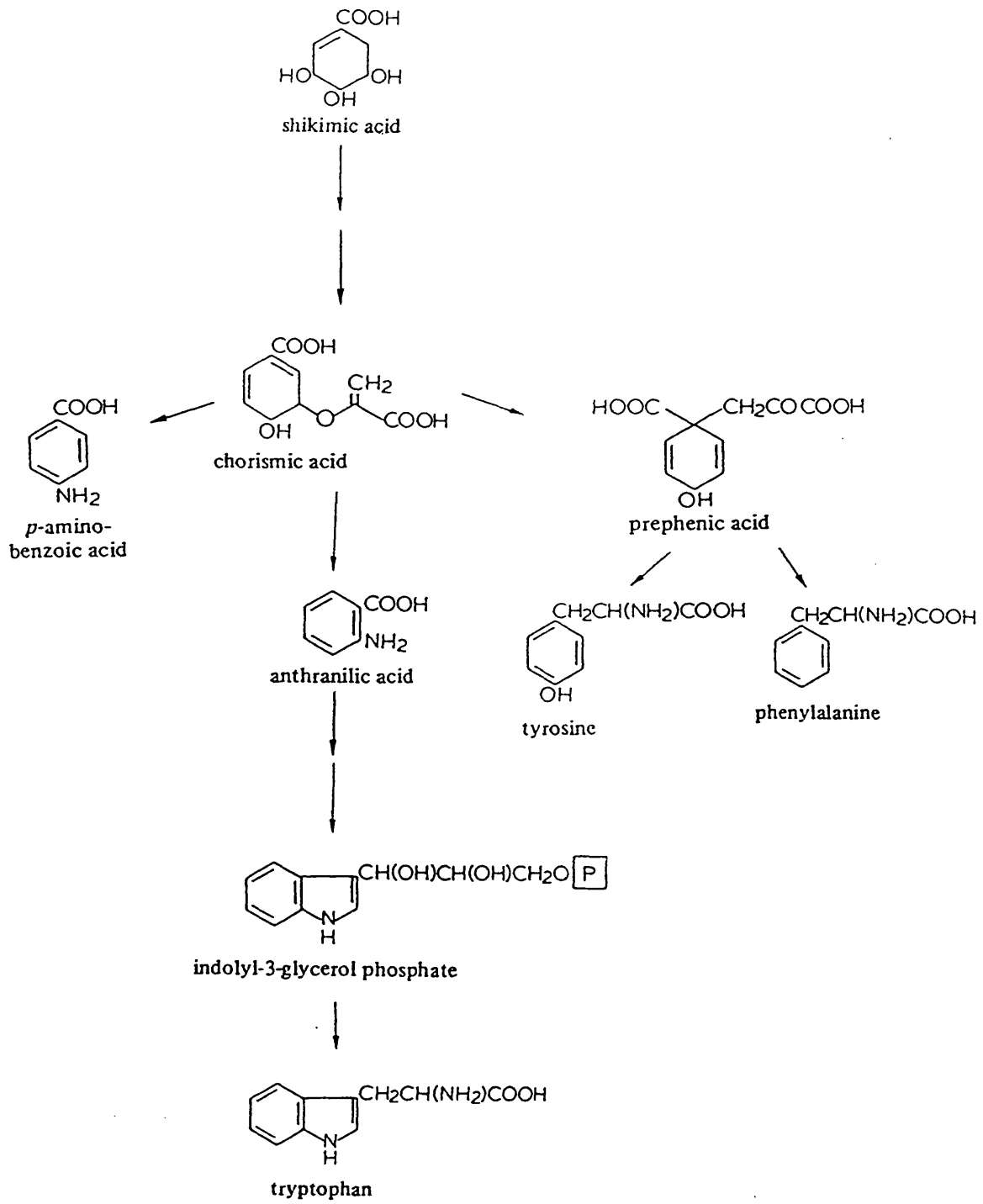


Fig. 1.2. : The shikimic acid pathway leading to tryptophan (Adapted from Vickery and Vickery, 1981).

Bialek, 1984), three biosynthetic routes, named after the key intermediate of each pathway, have been proposed (Reinecke and Bandurski, 1988; Sandberg *et al.*, 1987; Schneider and Wightman, 1974 & 1978). These are described in the following sections and shown in **Figs. 1.3-1.5.**

(a) Indole pyruvic acid-tryptamine pathway

The main pathways of IAA biosynthesis proceed from tryptophan to IAA via indole-3-acetaldehyde (IAAld, **6**) (Sembdner *et al.*, 1980) with one of two different intermediates, indole-3-pyruvic acid (IPyA, **7**) or tryptamine (**8**). This pathway is shown in **Fig. 1.3.**

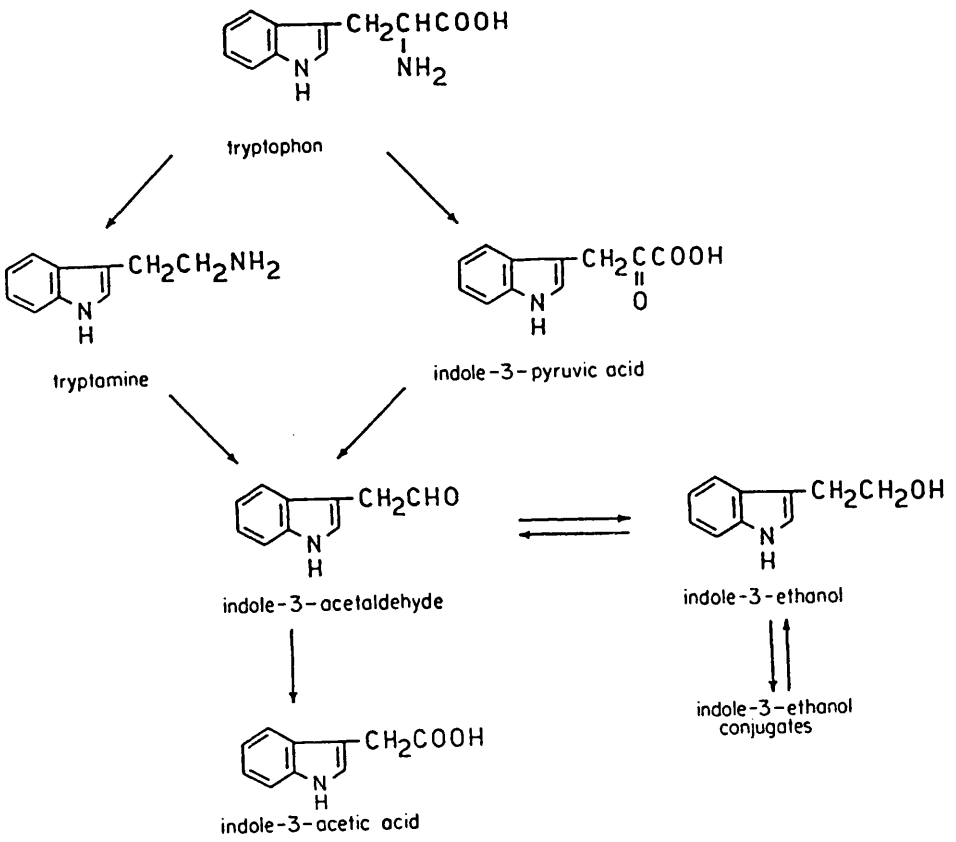


Fig. 1.3. Biosynthesis of IAA from tryptophan via tryptamine and indole-3-pyruvic acid (Reproduced from Sandberg *et al.*, 1987).

Although the instability of IPyA has made its identification as an endogenous compound difficult (Kaper and Veldstra, 1958), it appears that the IPyA pathway is the predominant route of synthesis in higher plants (Sandberg *et al*, 1987; Schneider and Wightman, 1978). The pathway involves the deamination of tryptophan to form IPyA which undergoes decarboxylation to yield IAAld, and is then converted to IAA.

The tryptamine pathway involves decarboxylation of tryptophan followed by oxidative deamination to IAAld. It appears from *in vitro* studies, that tryptophan decarboxylation is uncommon in plants (Sandberg *et al.*, 1987), although it has been detected in some cell-free preparations (Gibson *et al.*, 1972).

In addition to these pathways leading directly to IAA, there are a number of metabolic 'side shunts'. Indole-3-ethanol (IEt, **9**) and indole-3-lactic acid (ILA, **10**), reversibly formed from IAAld and IPyA respectively, appear to be the products of two such shunts (Schneider and Wightman, 1978) and are assumed to be storage forms possibly involved in the regulation of IAA biosynthesis and homeostasis (Berry *et al.*, 1989; Schramm *et al.*, 1987; Sembdner *et al.*, 1980; Reinecke and Bandurski, 1988).

(b) Indole acetaldoxime-indole acetonitrile pathway

Indole-3-acetaldoxime (IAOx, **11**) is converted to IAA via IAAld or transformed to indole-3-acetonitrile (IAN, **12**) which is then metabolised to IAA. In some members of the Brassiceae family, indole-based glucosinolates also act as IAA precursors (Schneider and Wightman, 1978). In this pathway, which is regarded as a side branch of the indole acetaldoxime-indole acetonitrile pathway, IAOx is converted via several steps to indole-3-methylglucosinolate (glucobrassicin, **13**) which can serve as a source of IAN (**Fig. 1.4.**).

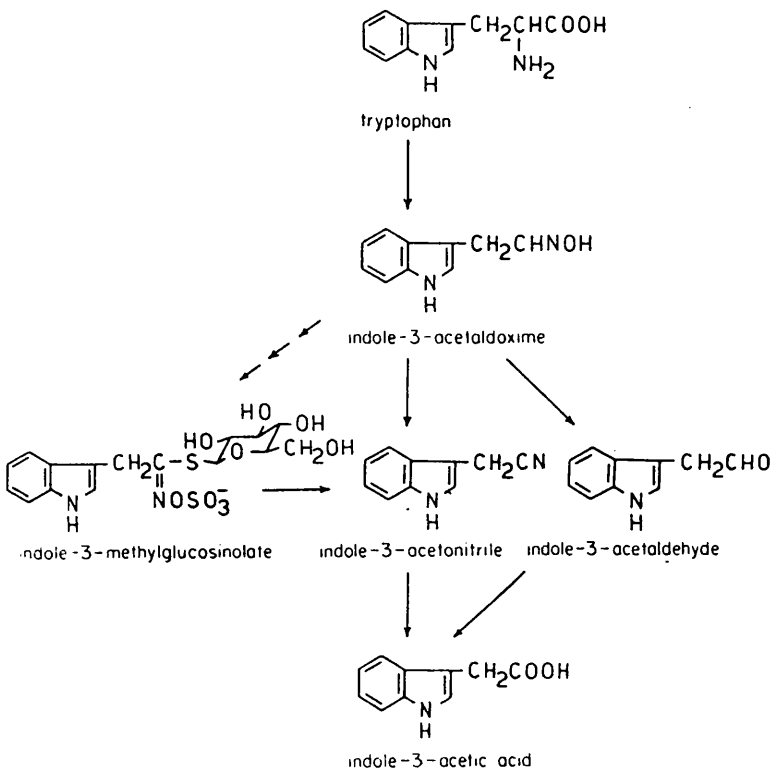
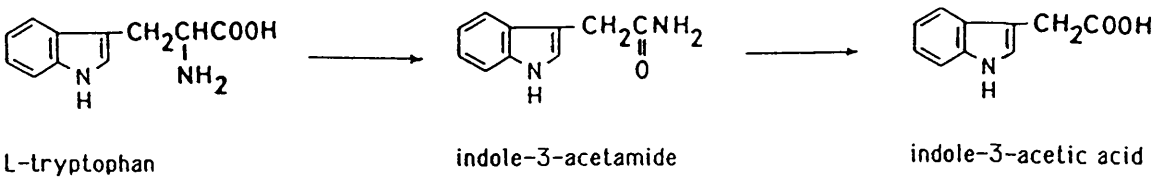


Fig. 1.4. Biosynthesis of IAA from tryptophan via indole-3-acetaldoxime (Reproduced from Sandberg *et al.*, 1987).

(c) Indoleacetamide pathway

IAA biosynthesis by plant pathogens, *Agrobacterium tumefaciens* (Nester *et al.*, 1984) and *Pseudomonas syringae* pv. *savastanoi* (Kosuge *et al.*, 1966), and by the symbiotic bacterium, *Bradyrhizobium japonicum* (Sekine *et al.*, 1988) have been shown to proceed from tryptophan to IAA via indole-3-acetamide (IAAm, **14**). Tryptophan undergoes oxidative decarboxylation to form IAAm which is then converted by hydrolysis to IAA. This pathway, which has not yet been found to operate in any plant species, is shown in **Fig. 1.5.**



L-tryptophan indole-3-acetamide indole-3-acetic acid

Fig 1.5: Biosynthesis of IAA from tryptophan via indole-3-acetamide (Kosuge *et al.*, 1966).

1.1.3. Degradation of IAA

(a) IAA catabolism

Catabolism of IAA in higher plants occurs as a result of oxidation, both decarboxylative, involving loss of the acidic side-chain carboxyl group and non-decarboxylative, where the C-1' carboxyl group is retained (Schneider and Wightman, 1974).

Decarboxylative oxidation leads either to the production of decarboxylated indoles i.e. indole-3-methanol (IM, **15**), indole-3-aldehyde (IAld, **16**) or indole-3-carboxylic acid (ICA, **17**) or to decarboxylated oxindoles such as oxindole-3-methanol (OxIM, **18**), 3-methyleneoxindole (MnO, **19**) and 3-methyloxindole (MO, **20**) (Sandberg *et al.*, 1987; Bandurski and Nonhebel, 1984) (**Fig. 1.6**).

Non-decarboxylative oxidation has been examined most thoroughly in *Zea mays* where the major product of IAA catabolism is oxindole-3-acetic acid (OxIAA, **21**) (Bandurski, 1984). OxIAA is further converted to 7-hydroxyoxindole-3-acetic acid (7-OH-OxIAA) which is then conjugated with glucose via the 7-hydroxyl group to yield 7-(*O*-ß-glucosyl)-oxindole-3-acetic acid (7-Gluc-OxIAA, **22**) (Bandurski and Nonhebel, 1984; Nonhebel and Bandurski, 1984).

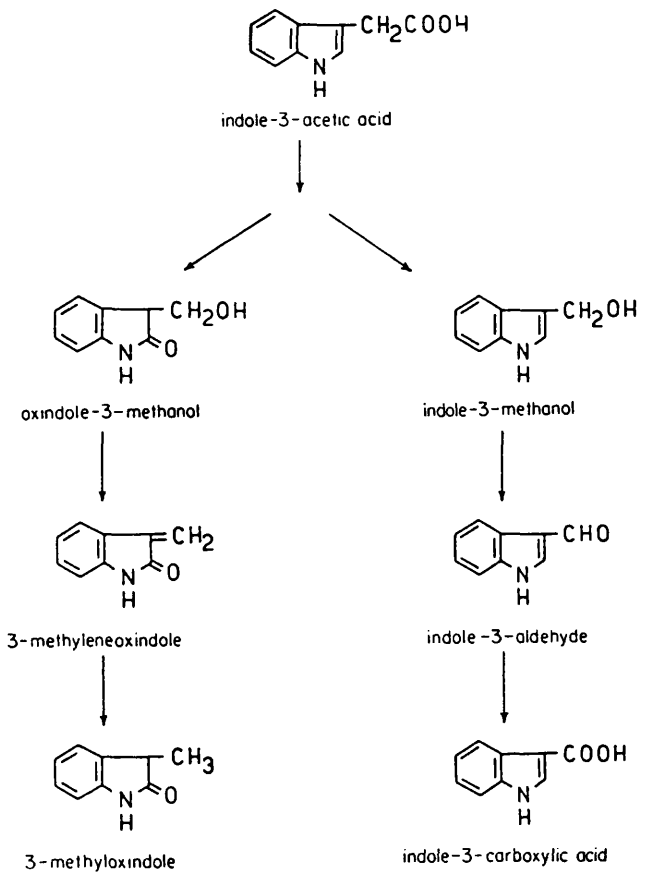


Fig. 1.6. Possible routes of decarboxylative IAA catabolism leading to decarboxylated oxindoles or decarboxylated indoles (Reproduced from Sandberg *et al.*, 1987).

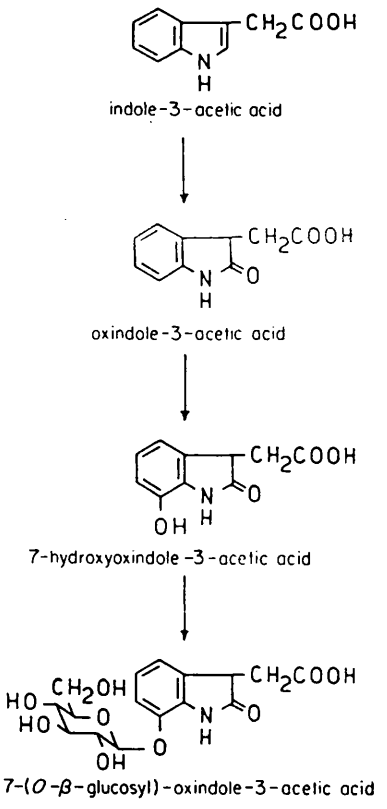


Fig. 1.7. Non-decarboxylative catabolism of IAA to 7-(*O*-ß-glucosyl)-oxindole-3-acetic acid in *Zea mays* (Reproduced from Sandberg *et al.*, 1987).

(b) IAA conjugation

Apart from altering the rates of biosynthesis, catabolism and formation of IAA storage compounds, the other main strategy for regulating IAA levels in plants is via the formation of IAA conjugates. It appears that most of the IAA found in plant tissues and seeds is in a conjugated form (Cohen and Bialek, 1984). These conjugates are produced by the covalent linkage, via a peptide, glycosidic or ester bond, of the IAA carboxyl group to another small molecule such as a sugar or amino acid. There are a range of conjugates and these have been classified into four main groups:

1. chlorinated auxins e.g. 5-chloro-IAA
2. glucosinolates e.g. IAA glucosyl ester

3. amino acid conjugates e.g. indole-3-acetylaspartic acid (IAAsp, **23**)

4. IAA-*myo*-inositols e.g. indole-3-acetyl-2-*myo*-inositol

Formation of sugar conjugates appears to be the major process of auxin metabolism in monocots (Sandberg *et al.*, 1987), while in dicots the main conjugate is IAAsp (Cohen and Bandurski, 1978). The production of such compounds is probably physiologically significant (Cohen and Bandurski, 1978); the compound shows significant auxin activity in several bioassays and is therefore not simply an inactive storage product. It is not known whether this activity is due to the molecule itself or to the hydrolytic release of IAA, since it is known that conjugation is reversible and that IAA is liberated by hydrolysis of the ester or amide linkages (Cohen and Bialek, 1984).

1.2. General introduction to *Rhizobium*

Members of the genus *Rhizobium* are non-photosynthetic, rod-shaped, Gram-negative bacteria which can live both free in the soil and symbiotically in the roots of certain leguminous plants in highly organised structures known as nodules. These nodules provide the necessary conditions for activity of the nitrogen-fixing enzyme, nitrogenase, responsible for converting free atmospheric nitrogen into ammonia. **Fig. 1.8.** presents a summary of the action of this enzyme. The ammonia produced is assimilated via a number of routes by the plant, eventually being incorporated into amino acids and proteins (Dixon and Wheeler, 1986). This readily available source of fixed nitrogen enables legumes to grow on soils which are deficient in nitrogen. Globally, this is responsible for the fixation of approximately 10^8 tonnes of N_2 *per annum* (Subba Rao, 1977; Downie and Johnston, 1988).

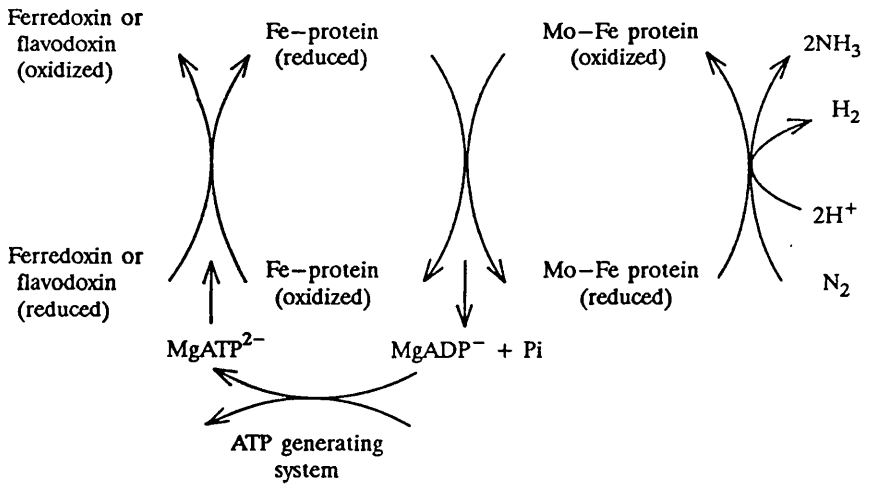


Fig. 1.8. Diagram summarising the nitrogenase reaction.

Reducing equivalents are passed through the enzyme complex via an iron-sulphur centre (Fe-protein) and iron-molybdenum centre (Mo-Fe protein) resulting in the subsequent reduction of the substrate, atmospheric N_2 (Adapted from Sprent and Sprent, 1990).

The importance of legumes

There are a number of reasons why it is likely that the importance of legumes as major food crops will continue to increase. As they are known to be excellent sources of protein, increased cultivation and productivity of leguminous species will be a necessity as the world fails to meet its total protein requirement from animal protein alone. In addition, in developing countries, the prohibitive cost of importing expensive nitrate fertiliser highlights the obvious economic advantage of growing a crop that is self-sufficient in nitrogen. At the other end of the spectrum, those countries able to afford fertilisers are faced with increasing environmental concern over the possible dangers to human health of nitrates in the water supply and by the eutrophication of seas and rivers (Subba Rao, 1977).

Taxonomy

Rhizobium is a member of the Rhizobiaceae, a family of bacteria which also includes the genera, *Agrobacterium*, *Bradyrhizobium* (formerly classified as slow-growing *Rhizobium* species [Jordan, 1982]), *Phyllobacterium* (Krieg and Holt, 1984) and *Azorhizobium* (Dreyfus *et al.*, 1988). It is now generally accepted that *Rhizobium leguminosarum*, *R. trifolii* and *R. phaseoli* are not distinct species but actually biovars of *R. leguminosarum* (Pinero *et al.*, 1988). Thus bacteria nodulating beans are classified as *R. leguminosarum* bv. *phaseoli* which, for convenience, will be referred to as *R. phaseoli* in this thesis.

1.3. Infection and nodulation by *Rhizobium*

1.3.1. The infection process

Nodulation is a very complex and specific series of events requiring the controlled, co-ordinated expression of both bacterial and plant genes. It can be divided into two broad stages, firstly, there are the early nodulation events, i.e. the infection process *per se*, which are followed by the development of the mature nodule (Libbenga and Bogers,

1974; Sprent, 1984). **Fig. 1.9.** illustrates the process of infection and nodulation in leguminous associations.

In brief, nodulation begins with bacterial multiplication and colonisation of the rhizosphere. After recognition and specific attachment of the bacterium to the host root hair cell, it is then possible for infection to take place via a process of root hair curling/deformation (Dixon and Wheeler, 1986). This curling is believed to facilitate infection by providing a closed system in which the enzymes necessary for cell wall penetration are prevented from diffusing away and therefore, at this localised site, the bacterium dissolves the root hair cell wall (Callaham and Torrey, 1981). Near the tip of the curled hair, there is an in-growth of the plant cell wall to form a tube, the infection thread, which acts to conduct the bacteria into the plant. The bacteria divide within this infection thread but although the bacteria are within the root, as the infection thread wall is plant cell wall and it, in turn, is surrounded by the plasmalemma, the bacteria remain extracellular. The infection thread grows down the root hair, preceded by the plant cell nucleus, and, contemporaneously proliferation of the inner cortical host root cells in the plant is induced initiating a new nodule meristem (Libbenga and Harkes, 1973). The thread then ramifies into the nodule cells where the rhizobia are subsequently released by a 'pinching off' process in which they are surrounded by a membrane of plant origin. The bacteria within this membrane are termed bacteroids and differ from the free-living forms of *Rhizobium* by being larger, pleiomorphic and capable of fixing nitrogen. The fully functional nodule is complete after differentiation of the nodule tissue and the synthesis and deposition in the cytoplasm, of leghaemoglobin, a plant-produced protein essential for the normal functioning of the nitrogenase enzyme. Ammonia, the product of fixation, is assimilated and used in the synthesis of organic nitrogenous compounds (Sprent, 1979; Sprent, 1984; Dixon and Wheeler, 1986).

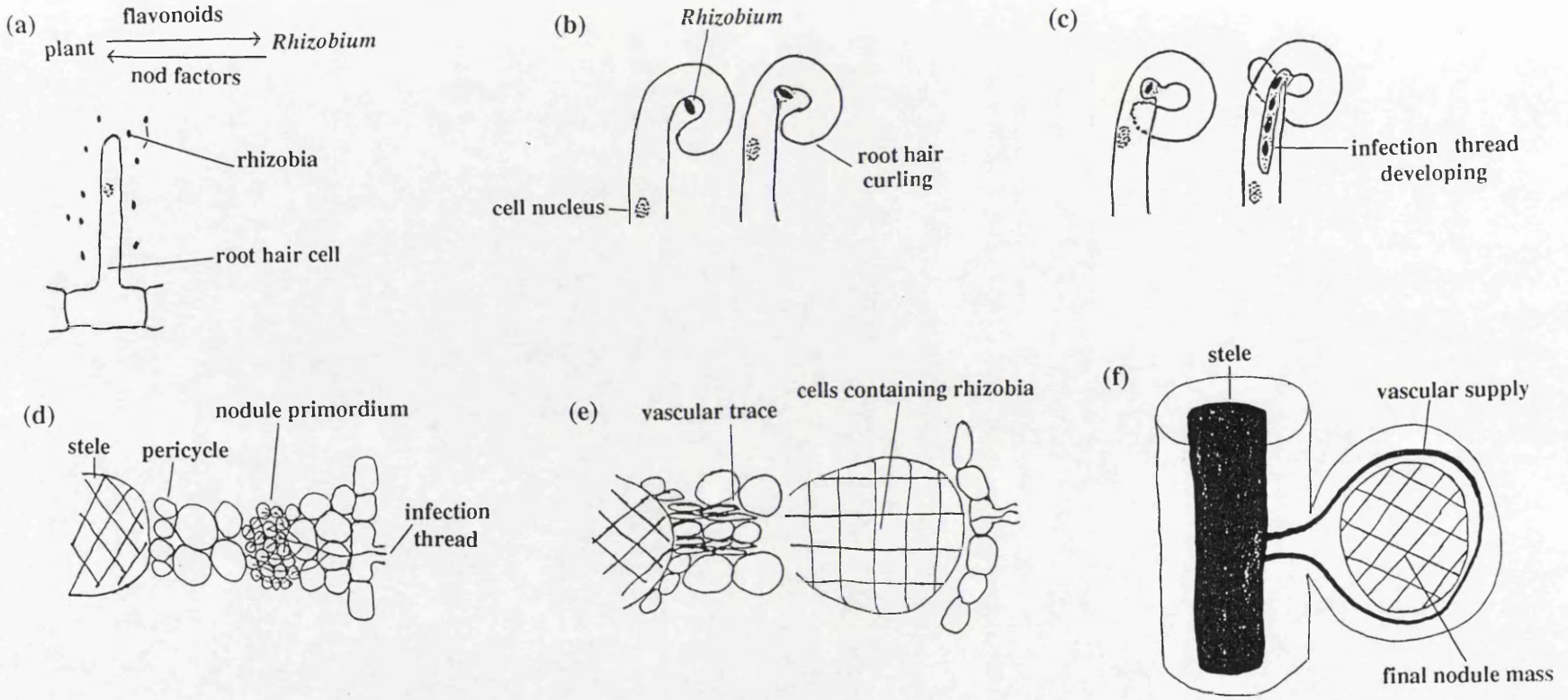


Fig. 1.9. Sequence of nodule development

(a) Plant production of flavonoids results in the activation of rhizobial *nod* genes, which encode Nod products (including oligosaccharides), that are responsible for root hair curling (b). After recognition, the *Rhizobium* cell binds to the root hair (b). (c) The *Rhizobium* cell invades the root hair cell and an infection thread develops. The cortical cells proliferate ahead of the advancing thread which culminates in the formation of the nodule primordium (d). The bacteria are released from the thread and the cells containing the rhizobia divide and enlarge and a vascular supply develops (e). The nodule is mature after the bacteria differentiate into bacteroids (f).

1.3.2. Specificity of nodulation

An important feature of the *Rhizobium*-legume symbiosis is that it is highly specific, particular legumes being infected only by a limited range of rhizobial strains or species. This implies a high degree of specificity in the process of recognition.

The mechanism of specificity is complex and much remains to be learned about the mechanisms involved. However, it is clear that both plant and bacterial genetic determinants are involved in the specificity of the nodulation process (Brewin *et al.*, 1980). It has been found that legumes belonging to one cross-inoculation group are nodulated only by certain classes of *Rhizobium* and indeed *Rhizobium* spp. are defined in terms of the host legumes that they are able to nodulate (Johnston *et al.*, 1978). Studies with mutant species of *Rhizobium* have demonstrated that the bacterial determinants for formation of N-fixing nodules on the host legume reside on plasmids which carry the information required for symbiosis and are also believed to determine the specificity of nodulation. These large symbiotic or *Sym* plasmids, contain the loci, *nif* and *fix,* which are involved in N_2-fixation, nitrogenase activity and expression, as well as the *nod* genes, which are involved in the instigation of nodule formation (Downie and Johnston, 1988). Thus by transfer of the *Sym* plasmid from a wild-type strain of *R. leguminosarum* (pea-nodulating), the ability to nodulate peas was conferred to a non-nodulating strain of *R. leguminosarum* and to three other species of *Rhizobium* that previously nodulated legumes other than peas. Beynon *et al.* (1980) found that when the *Sym* plasmid was deleted in *R. phaseoli* the ability to nodulate *Phaseolus* beans was abolished and when this plasmid was subsequently transferred to *R. leguminosarum,* the bacteria were able to nodulate both peas and *Phaseolus*. From these results it was possible to infer that at least some of the information needed for nodulation and specificity is plasmid-linked (Brewin *et al.*, 1980).

1.3.3. Plant and bacterial recognition

The initial nodulation steps of the *Rhizobium*-legume symbiosis can be thought of as a two-way molecular conversation (Fisher and Long, 1992). The initial signalling of *Rhizobium* by the host legume involves the exudation from the roots of low molecular weight flavonoids, with skeletal or side group modifications to distinguish the inducers from distinct plants (Downie and Johnston, 1988). These induce the expression of bacterial genes required for nodulation (*nod* genes) by binding specifically to a gene, *nod*D, which causes the other *nod* genes to be activated (Fisher and Long, 1992).

These *nod* genes, in turn, encode enzymes involved in the synthesis of Nod factors which cause morphological changes in the plant root hair. It is known that these rhizobial *nod* gene products are essential for root hair deformation and cortical cell division and that *nod*$^-$ strains are unable to initiate the formation of nodules (Downie and Johnston, 1988). Since the completion of this research, it has been discovered that a *nod* gene product, NodRm-1, a purified sulphated and acylated glucosamine oligosaccharide from *R. meliloti* elicited genuine root-nodule organogenesis on aseptically grown alfalfa (Truchet *et al.*, 1991). The *nod*ABC genes, common to all *Rhizobium* species and the host specific genes *nod*H and *nod*PQ were involved in the production of this compound. As the *nod*ABC operon is expressed in the infection thread close to which cortical cells are dividing, and as the Nod factors also stimulated plant expression of the 'early nodulins' (host proteins expressed during the initial response to rhizobial infection), Truchet *et al.* (1991) postulated that NodRm-1 is involved not only in the induction of root hair deformation and curling, but also in the initiation of the first rounds of cortical cell division and in nodule organogenesis. It has been discovered recently that Nod factors from other species are also substituted oligosaccharides with different host-specific modifications (Fisher and Long, 1992).

At present, it is not known which plant systems the Nod factors interact with. Nodule morphogenesis, including early nodulin gene expression is mimicked by auxin transport inhibitors and by cytokinins (Hirsch *et al.*, 1989). Nodule growth therefore could be caused by changes in root auxin or cytokinin levels, transport, or sensitivity.

1.3.4. Rhizobial attachment

After signalling, the bacteria become specifically attached to the root hair surface, possibly by interaction with lectins produced by the plant and cellulose microfibrils produced by the bacterium (Sequeira, 1978; Dazzo and Truchet, 1984). Additionally, Smit *et al.* (1989) isolated and purified a Ca^{2+}-dependent adhesin from *R. leguminosarum* bv. *viciae* believed to mediate the first step in attachment of *Rhizobium* cells to the root hair tips. This rhicadhesin is located on the cell surface of the bacterium and was found to be produced by all members of the Rhizobiaceae (Smit *et al.*, 1989).

1.4. IAA production by *Rhizobium in vitro* and *in planta*

1.4.1. IAA and root hair infection

It was suggested as far back as the 1930's (Thimann, 1936; Link, 1937, Kefford *et al.*, 1960) that IAA produced by *Rhizobium* could be a regulator of the initiation and formation of root nodules, particularly with regard to the phenomenon of root hair curling. Appealing as this theory might be, it seems that curling is not simply a consequence of bacterial IAA production. There are several lines of evidence which have led to this theory falling from favour.

Firstly, many bacteria produce IAA but have no corresponding effect on legume root hairs (Morris, 1986). Secondly the 'curling factor' is known to be heat stable up to 100°C and have poor diffusion properties. These eliminate IAA as the causal agent as it does not exhibit these properties (Yao and Vincent, 1976). Thirdly, it has been found that

nod- mutant strains of *R. leguminosarum* which are unable to cause root hair deformation (although able to adhere to the root hair) are still capable of producing IAA (Badenoch-Jones *et al.*, 1982b). Atzorn *et al.* (1988) found that IAA excretion was a common property of all the *R. phaseoli* strains they tested, including mutants defective in nodulation and nitrogen fixation. Wang *et al.* (1982) also failed to find a correlation between the ability of *nod*- mutants (which had been cured of their nodulation plasmids) and wild-type *R. leguminosarum* to nodulate peas and the ability to produce IAA. It was found however that wild-type strains consistently produced slightly more IAA than the non-nodulating mutant strains. Most recently, as detailed in **1.3.3.**, the formation of genuine nodules on alfalfa by a purified *nod* gene product, in the absence of *R. meliloti* (Truchet *et al.*, 1991), would seem to rule out IAA involvement in the early stages of infection.

1.4.2. Involvement of IAA in the late stages of infection and nodulation

Although IAA does not appear to be involved in the initial root hair curling and infection (**1.4.1.**), there is evidence that phytohormones are involved in subsequent stages of nodulation. It is possible, for example, that IAA is involved in the release of the bacteria from the infection thread, in the maintenance of the nodule to prevent tissue senescence or in some aspect of N-fixation.

It has been found that levels of IAA (Dullaart, 1967) and cytokinins (Phillips and Torrey, 1970) in legume nodule tissue are higher than those found in uninfected roots. Accordingly, it has been postulated that the balance of these phytohormones in infected tissue has an important role in the nodulation process (Dullaart, 1970; Wang *et al.*, 1982). Indeed, application of auxin and cytokinin to explants of root cortical tissue results in a pattern of proliferation similar to the initial proliferative stages of root nodule formation *in vivo*. Consequently, it was speculated that the bacterium may initiate nodule development by altering the auxin/cytokinin ratio of root cortical cells resulting in cell

division (Libbenga *et al.*, 1973). It may be that *Rhizobium* controls the auxin/cytokinin ratio by producing inhibitors of auxin synthesis, transport or action. IAA may be involved in the structural changes which take place in the root hair wall, as IAA has long been associated with cell wall loosening and growth (Theologis, 1986).

Nodule morphogenesis, including early nodulin gene expression, is mimicked by auxin transport inhibitors. Hirsch *et al.* (1989) found that application of synthetic auxin transport inhibitors, [N-(1-naphthyl)phthalamic acid and 2,3,5-triiodobenzoic acid] caused the induction of nodule-like structures (pseudonodules) on the roots of alfalfa. The histology of these structures closely resembled those of *Rhizobium*-induced nodules. These results suggested that the change in the auxin/cytokinin ratio of the tissue, led to cellular division and the production of these pseudonodules.

1.4.3. Location of IAA genes

One may compare the results of Wang *et al.* (1982), who found that wild-type strains of *R. leguminosarum* consistently produced slightly more IAA than non-nodulating mutant strains, with those found in oncogenic and non-oncogenic (Ti-plasmid absent) strains of *Agrobacterium tumefaciens* where although both strains produced IAA, production in the non-oncogenic strains was much lower. This led to the suggestion that there are two pathways of auxin biosynthesis in *A. tumefaciens*; one chromosomal and one plasmid-borne (Liu *et al.*, 1982). It has been proposed that an analogous situation may exist in *Rhizobium.*

The genes for IAA biosynthesis in *Rhizobium,* although unlocated at present, do not appear to reside on the large *Sym* plasmid (Badenoch-Jones *et al.*, 1982b). This has been backed up by the work of Atzorn *et al.* (1988) who suggested that it is unlikely that the IAA and GA biosynthetic genes are closely linked to the genes concerned with either nodulation or N_2-fixation.

1.4.4. IAA levels in nodules

The level of a particular plant growth regulator in a tissue/organ depends on several key factors. **Table 1.1.** illustrates the factors which determine the level of IAA present in legume root nodule tissue.

Increase in level	Decrease in level
***de novo* synthesis**	**degradation**
transport inward	**transport outward**
release from conjugate	**conjugate formation**

Table 1.1. Factors regulating the balance of IAA in root nodules (Reinecke and Bandurski, 1988).

(a) Import and export

One question that should be asked when trying to elucidate the rôle of auxin in the nodule is the precise origin of the IAA involved. It is known that nodule tissue has a higher level of IAA than surrounding uninfected root (Dullaart, 1970; Badenoch-Jones *et al.*, 1983). How can these higher IAA levels be explained?

It is believed that a substantial part of the IAA in the nodules is produced by the nodules themselves (Badenoch-Jones *et al.*, 1984). However, radiolabelling of the apical bud of *Pisum sativum* with [3H]-IAA showed clearly that IAA is imported into the nodule where it accumulates (Badenoch-Jones *et al.*, 1983). It appears that the bacteroids themselves have an major influence over this inward flow of IAA as the amount of radioactivity which accumulates in the nodule is far more than that which accumulates in the root tissue. It was also found that accumulation was higher in nitrogen-fixing (effective) than in non-nitrogen-fixing (non-effective) nodules (Badenoch-Jones *et al.*, 1983). This difference in the level of IAA between effective and non-effective nodules

adds weight to the theory that the metabolism of auxins may be important for the persistence of a functional nodule.

Additional work by Badenoch-Jones and her colleagues (1984) have revealed that nodule tissue not only acts as a sink, but perhaps also as a source of IAA. Application of [^{3}H]-IAA to the root nodules *in situ* demonstrated the rapid export of IAA to all plant parts.

(b) IAA synthesis in the nodule

There have been few studies of endogenous indole compounds in root nodules. Dullaart (1970) analysed the root and nodule extracts of *Lupinus luteus* by two-dimensional thin-layer chromatography (TLC) and detected IAA, ICA, IAld, ILA and indole-3-glycolic acid (IGA). Badenoch-Jones *et al.* (1984) examined the endogenous indoles found in pea nodules by GC-MS and detected IAA, IAAsp, IPyA, ILA, IPA, IBA, ICA and IM.

Dullaart (1970) suggested that the higher levels of IAA in nodule tissue could be due to an alteration of indole metabolism in the tissue itself induced by rhizobial infection. It was found that the ICA content of *Lupinus* parental roots was three times higher than that of the nodule tissue. It is possible that the different levels of ICA in root and nodule tissue are due to altered rates of IAA degradation. Similar changes in metabolism are frequently found in diseased tissues. In extracts of normal healthy tomato stem tissue only ICA, a degradation product of IAA, is detected, while in tomato crown gall tissue both ICA and IAA are found (Sequeira, 1973).

As mentioned previously, this type of alteration in metabolism occurs when *Agrobacterium* transfers the T-DNA from the Ti-plasmid into the host cell genome. *Rhizobium* does have plasmids but there is no evidence as yet of the transformation of the nodule tissue by the infecting bacterium (Brewin *et al.*, 1980).

(c) The catabolism of nodule IAA

There are three possible fates of IAA in nodule tissue; it may accumulate or be degraded (catabolised) or joined to another molecule (conjugated). These processes are believed to play an integral part in the homeostatic control of IAA levels. Application of [^{3}H]-IAA to *Pisum sativum* root nodules and subsequent analysis by TLC and GC-MS, revealed its rapid conversion to the IAA conjugate, IAAsp, which was also the main endogenous indole (Badenoch-Jones *et al.*, 1984). The precise physiological significance of the production of IAAsp is not understood although conjugation is generally viewed as being a mechanism for maintaining homeostasis, enabling a tissue to accumulate IAA without causing major changes in the steady-state concentration of free IAA.

1.4.5. Indole production by *Rhizobium* in culture

(a) Indoles detected in rhizobial culture medium

It was postulated that production of IAA by *Rhizobium* could be responsible for the high IAA content of nodules and indeed studies have shown that IAA is produced from tryptophan by several species of *Rhizobium* in culture: *R. leguminosarum* (Badenoch-Jones *et al.*, 1983), *R. meliloti, R. lupini* (Dullaart, 1970) and *R. phaseoli* (Ernstsen *et al.*, 1987). **Table 1.2.** lists the spectrum of indolic compounds found by Badenoch-Jones and her collaborators (1982a) when they carried out a gas chromatography-mass spectrometry (GC-MS) study on *R. leguminosarum* bv. *leguminosarum* culture medium and achieved the first unequivocal identification of rhizobial IAA production. More recently Ernstsen *et al.* (1987) examined the indole production of *R. leguminosarum* bv. *phaseoli,* grown in non-tryptophan supplemented media, using high performance liquid chromatography (HPLC) and GC-MS.

As can be seen from **Table 1.2.**, there are differences in the spectrum of indoles when the biovars are compared. These differences could be due to variations between rhizobial

Indolic compound	*R. leguminosarum*	
	bv. *leguminosarum*	bv. *phaseoli*
IAA	+	+
IAld	+	+
IEt	+	+
ICA	+	ND
IGA	+	ND
ILA	+	ND
IGoxA	+	ND
IPyA	+	ND
IM	ND	+
N-acetyl-L-tryptophan	+	ND
Tryptamine	ND	ND

+ detected **ND not detected**

Table 1.2. The profile of indolic compounds detected in two biovars of *Rhizobium leguminosarum.*

The indoles produced by *R. leguminosarum* bv. *leguminosarum* in tryptophan-supplemented medium (56 mg l^{-1})(Badenoch-Jones *et al.*, 1982) and by *R. leguminosarum* bv. *phaseoli* in tryptophan minus medium (Ernstsen *et al.*, 1987).

strains or they could be a consequence of the addition of tryptophan to the medium of *R. leguminosarum*, resulting in a higher production of IAA and thus an enhancement of the levels of indolic intermediates and catabolites. Alternatively, this may be an indication that there are metabolic routes which are restricted to tryptophan-enriched cultures. The stimulation of IAA production by the addition of tryptophan has been well documented, however alternative biosynthetic routes have not, as yet, been investigated.

(b) IAA biosynthesis by *Rhizobium*

The biosynthetic pathway of IAA production by *Rhizobium* has not yet been demonstrated conclusively. Dullaart (1970) analysed the indolic compounds of *R. lupini* culture medium by thin-layer chromatography (TLC). Although he was unable to detect IPyA, he hypothesised that IPyA is probably the first intermediate between tryptophan and IAA in the bacterial production of IAA and his lack of success in isolating the compound was perhaps due to its instability during extraction. Nevertheless demonstration that the production of IAA from tryptophan *in vitro* is stimulated by the addition of pyridoxal phosphate and alpha-ketoglutarate points to involvement of a transamination reaction in the conversion which adds further weight to Dullaart's theory. Ernstsen *et al.* (1987) proposed that in *R. phaseoli* strain 8002, the pathway from tryptophan to IAA is via IPyA and IAAld (**Fig. 1.10.**).

Tryptophan → IPyA → IAAld → IAA

Fig 1.10. Possible pathway of IAA biosynthesis from tryptophan via IPyA and IAAld.

Both Badenoch-Jones *et al.* (1982) and Ernstsen *et al.* (1987) failed to detect IPyA and IAAld; this is possibly due to the fact that both of these molecules are highly unstable and as such, tend to break down readily during purification (Kaper and Veldstra, 1958).

IAAm was not detected as either an endogenous constituent, even though it is a fairly stable compound (Badenoch-Jones *et al.*, 1982a; Ernstsen *et al.*, 1987), or as a metabolite of [^{3}H]-tryptophan in *Rhizobium* culture (Ernstsen *et al.*, 1987). Nor do cultures convert [^{14}C]-IAAm to IAA (Ernstsen *et al.*, 1987). The IAA biosynthetic pathway operating in *Rhizobium* is thus clearly different from the one elucidated for *Pseudomonas* and *Agrobacterium* in which IAAm is the primary intermediate (Morris, 1986) (**1.5.**). Moreover, the IAA biosynthetic pathway in the slow-growing rhizobial genus, *Bradyrhizobium* has been investigated by Sekine *et al.* (1988) who suggested that IAA is produced from tryptophan via IAAm. IAAm hydrolase activity was detected in *Bradyrhizobium* but not in any of the *Rhizobium* species tested (**1.5.**).

(c) IAA catabolism by *Rhizobium in vitro*

Ernstsen *et al.* (1987) found that IAA is catabolised to IM. This occurs via a decarboxylative pathway involving loss of the 1' carbon. As ICA has been detected in *Rhizobium* cultures (Badenoch-Jones *et al.*, 1982a), they further speculated the final product would be ICA produced via IAld, although the final two steps have not, as yet, been demonstrated.

The catabolism of IAA by *Rhizobium* therefore appears to be decarboxylative. This differs from the situation in higher plants where IAA catabolism is thought to be primarily non-decarboxylative (Bandurski and Nonhebel, 1984).

1.4.6. IAA production by bacteroids

(a) Bacteroid synthesis of IAA

It has been shown that *Rhizobium* produces IAA *in vitro* however the situation *in planta* i.e. in the nodule, remains uncertain. The metabolism of *Rhizobium* in the bacteroid form is different from that of the free-living bacterium, and thus the pathway of IAA production *in vitro* may not be the same as in the bacteroid tissue. Differences between

indole metabolism by the free-living bacterium and bacteroid could be used potentially to discriminate between the relative contributions of the microsymbiont and the host plant to the overall process of IAA metabolism in the root nodules.

Hunter (1989) showed that *Bradyrhizobium japonicum* can produce IAA in symbiotic association with the host legume. Bacteroids were isolated and it was found that they produced IAA from tryptophan, but did not degrade IAA. Bacteroids from high IAA-containing nodules had an enhanced ability to produce IAA, implying that bacteroid-produced IAA accumulates in the nodule.

(b) IAA catabolism in bacteroids

Rigaud and Puppo (1975) found considerable IAA-oxidase activity in preparations of soybean bacteroids (*Glycine max*). This oxidative degradation of IAA was reduced at low O_2 partial pressure. As these conditions are likely to occur in the nodules, they could therefore limit the rate of IAA degradation by the bacteroids. In line with this hypothesis, Hunter (1989) failed to detect IAA degradation in his *Bradyrhizobium* bacteroid preparations.

1.4.7. Summary

It is clear that the development and persistence of a functional root nodule requires a high degree of regulation. It is believed that hormonal balance plays an important rôle in this control. It could be the case that IAA activates certain genes which affect the biosynthesis of particular proteins at the transcriptional or post-transcriptional level; or switch on genes coding for polypeptides necessary for cell division, differentiation or adventitious meristem initiation. At the present time there is little evidence in support of these ideas but it is hoped that a thorough knowledge of the metabolism of indoles by the microsymbiont, both *in vitro* and *in planta,* and by the host itself will give an insight to the physiological basis of the symbiosis.

1.5. Other IAA-producing micro-organisms

1.5.1. Range of micro-organisms

In addition to *Rhizobium*, indolic compounds are produced by a number of other micro-organisms, some of which are phytopathogens, while others can exist in symbiosis with plants. Examples of micro-organisms known to be IAA-producers are listed in **Table 1.3.**

Although it appears that the majority of IAA-producing bacteria are plant pathogens, the possibility exists that the phenomenon of IAA production is widespread throughout the fungal and bacterial kingdoms and it is therefore a coincidence caused by the intensity of research on plant pathogens, which makes it appear that the majority of IAA-producers are pathogens. It is also interesting to note the difference in viewpoint, with regard to IAA and cytokinins, between plant physiologists who consider these to be 'plant growth regulators' and pathologists who frequently refer to them as 'pathogenicity factors'.

A number of these phytopathogens e.g. *Taphrina*, *Agrobacterium* and *Pseudomonas* cause hyperplasia and hypertrophy on their hosts elicited apparently as a result of hormonal imbalance, particularly of IAA and cytokinins. These abnormalities are seen as knots, galls and fasciations and are presumably the result of enhanced meristematic activity.

It is known in a number of these cases that IAA production is intimately associated with pathogenicity. The pathways of IAA biosynthesis in *Pseudomonas syringae* pathovar (pv.) *savastanoi* (Kosuge *et al.*, 1966) and *Agrobacterium tumefaciens* (Nester *et al.*, 1984) have been studied in detail and have been reviewed by Morris (1986).

Species	Status	Reference
Fungal species		
Endomycopsis vernalis	**N**	**1**
Pisolithus tinctorius	**E**	**2**
Phycomyces blakesleeanus	**N**	**3**
Rhizopus suinus	**N**	**4**
Taphrina deformans	**P**	**5**
Bacterial species		
Agrobacterium rhizogenes	**P**	**6**
Agrobacterium tumefaciens	**P**	**7**
Azospirillum brasilense	**M**	**8**
Azospirillum lipoferum	**M**	**9**
Bradyrhizobium japonicum	**S**	**10**
***Frankia* spp.**	**S**	**11**
Pseudomonas savastanoi	**P**	**12**
***Rhizobium* spp.**	**S**	**13**
Rhodococcus fascians	**P**	**14**

Table 1.3. Examples of IAA-producing micro-organisms

Status key : P-plant pathogen, **S**-capable of existing in symbiosis, **E**-endomycorrhizal fungus, **M**-mutualistic relationship and **N**-no known relationship with a plant.

References

1. Glombitza and Hartmann (1966) **2.** Frankenberger and Poth (1987) **3.** Schramm *et al.* (1987) **4.** Thimann (1935) **5.** Link *et al.* (1937), Perley and Stowe (1966) **6.7.** Kaper and Veldstra (1958), Liu and Kado (1979) **8.** Horemans and Vlassak (1985) **9.** Abdel-Salam and Klingmüller (1987) **10.** Kaneshiro and Kwolek (1985), Sekine *et al.* (1988) **11.** Berry *et al.* (1989), Wheeler *et al.* (1984) **12.** Kosuge *et al.* (1966) **13.** Badenoch-Jones *et al.* (1982a), Ernstsen *et al.* (1987) **14.** Chatterjee and Vidaver (1986).

1.5.2. *Pseudomonas syringae* pv. *savastanoi*

Pseudomonas syringae pv. *savastanoi* is a bacterial pathogen which infects olive, privet and oleander. The pathogen enters the host through wounds and causes knots/galls to appear on infected leaves and twigs. These provide the ideal environment for multiplication of the parasite during the hot, dry summers characteristic of the regions in which these plants grow (Nester and Kosuge, 1981).

Magie and Wilson (1962) identified IAA in the culture medium of the bacteria and suggested correctly that it was involved in gall formation. The first intermediate of IAA biosynthesis in *Pseudomonas savastanoi* is indole-3-acetamide (IAAm), formed from the oxidative decarboxylation of tryptophan by the enzyme tryptophan-2-monooxygenase. The IAAm is then hydrolysed to form IAA by IAAm hydrolase. Finally, the IAA is conjugated with lysine to form indole-acetyl-L-lysine (Kosuge *et al.*, 1966). Addition of tryptophan to the culture medium of the bacterium *in vitro* enhanced the accumulation of IAA, apparently by induction of the biosynthetic enzymes (Kuo and Kosuge, 1969). In oleander strain EW2009, two of the genes encoding IAA biosynthetic enzymes, tryptophan monooxygenase and indole acetamide hydrolase are borne on a 52 kb plasmid called pIAA1 (Comai and Kosuge, 1980). The genetic loci of these genes were mapped by molecular cloning and characterisation of insertion mutations (Comai and Kosuge, 1982) and were designated *iaaM* (tryptophan monooxygenase) and *iaaH* (IAAm hydrolase). The pathway of IAA biosynthesis and conjugation in *Pseudomonas syringae* pv. *savastanoi.* (Kosuge *et al.*, 1966) is summarised in **Fig. 1.11.**

Tryptophan→IAAm→IAA→Indole-acetyl-L-lysine
iaaM ***iaaH***

Fig. 1.11. IAA biosynthesis and conjugation in *Pseudomonas syringae* pv. *savastanoi.* (Kosuge *et al.*, 1966).

Comai *et al.* (1982) found that in two *Pseudomonas savastanoi* strains from oleander, deletion of the plasmid coding for IAA was accompanied by loss of virulence. If the plasmid was subsequently reintroduced into these *Iaa*$^-$ mutants, IAA production and virulence were restored. Thus IAA is encoded on a plasmid in these strains. In contrast to this, 10 strains examined from privet and olive retained their IAA production and virulence after removal of the plasmid DNA demonstrating that the genes of interest were chromosomal (Comai *et al.*, 1982).

Smidt and Kosuge (1978) isolated 3 spontaneous non-IAA producing mutants (*Iaa*$^-$) of *Pseudomonas savastanoi* and found that following vacuum infiltration into the host, they would grow but were unable to stimulate gall production. Additionally, they found that mutants with an increased capacity for IAA synthesis incited formation of more severe symptoms than the parental isolates.

In contrast to *Agrobacterium* (**1.5.3.**), there is no evidence that plant cells infected with *P. savastanoi* are transformed. In other words, knot formation appears to depend only on bacterial production of phytohormones and does not involve an exchange of genetic material.

1.5.3. *Agrobacterium tumefaciens*

Agrobacterium tumefaciens is the causative agent of crown gall disease which is seen as undifferentiated, hypertrophied tumours or as abnormal stunted shoots on a wide range of gymnosperms and dicotyledonous angiosperms. The bacterium is a member of the family Rhizobiaceae and is believed to be closely related to members of the genus *Rhizobium* (Krieg and Holt, 1984). However, in contrast to *Rhizobium* which produces highly differentiated root nodules, *Agrobacterium* induces only abnormal and tumourous cellular development of the host. When it was shown that crown gall tissue culture retained its tumourous properties, even in the absence of the bacterium, it became clear

that these plant cells had been altered permanently (White and Braun, 1942). In addition, secondary galls away from the initial infection site were found to be bacteria-free (Braun, 1958). These tissues had obviously been affected by a transmissible agent.

This phenomenon is now known to be the result of the stable covalent integration of a small piece of DNA, (transferred or T-DNA) which is carried on a large virulence plasmid (the tumour-inducing or Ti-plasmid) from the bacterium into the plant nuclear DNA (Chilton *et al.*, 1977). Once in place, this DNA is transcribed and translated by the genetic machinery of the plant.

It has been shown that the T-DNA encodes the enzymes that participate directly in the phytohormone biosynthesis which results in this abnormal tissue development (Chilton *et al.*, 1977; Liu and Kado, 1979). Liu *et al.* (1982) showed that the plasmid-borne genes for IAA production are absolutely essential for tumour induction. Unlike normal tissue, transformed tissues are able to grow autonomously in culture in the absence of exogenous auxin or cytokinin; presumably because they contain levels capable of promoting cell growth and division (Braun, 1958).

The pathway of IAA biosynthesis in crown gall tissue is via IAAm and is therefore identical to that operating in *P. savastanoi* (Nester *et al.*, 1984). This pathway is illustrated in **Fig. 1.12.** It is not known if IAA is conjugated to form indole-acetyl-L-lysine as in *P. savastanoi.*

Tryptophan → IAAm → IAA

tms1 ***tms2***

Fig. 1.12. Biosynthesis of IAA in *A. tumefaciens* crown gall tissue (Nester *et al.*, 1984). Garfinkel *et al.* (1981) identified one locus, the *tms* (tumour morphology shoot) locus which causes shooty tumours when mutated. Two genes (*tms1* and *tms2*) have been mapped at this locus.

The phytohormones produced, determined by the type of infecting Ti-plasmid, play a central role in influencing tumour morphology. *A. tumefaciens* produces either shooty teratomata (abnormal stunted shoots) or unorganised tumours. This is an indication that a differential synthesis of auxin and/or cytokinin is controlling tumour morphology in the same way that the development of callus tissue *in vitro* is controlled by the relative ratio of exogenous auxin and cytokinin application (Skoog and Miller, 1957). In tissue culture a high auxin to cytokinin ratio results in root formation, a low ratio in shoot development, while an intermediate ratio results in the production of undifferentiated callus.

Mutations in the T-DNA results in changes of tumour morphology. These have been reviewed by Nester *et al.* (1984) and Weiler and Schröder (1987). Three distinct loci have been pin-pointed from the study of Garfinkel *et al.* (1981): first, the *tms* (tumour morphology shoot) locus causes shooty tumours when mutated and is believed to be involved in auxin metabolism. Two genes (*tms1* and *tms2*) have been mapped at this locus. The second locus, *tmr* (tumour morphology root) causes root-forming tumours when mutated and is believed to be concerned with cytokinin production (one gene named *4* has been mapped) and the *tml* (tumour morphology large) locus causes tumours two to three times larger than wild-type when inactivated (Garfinkel *et al.*, 1981). At present the function of the *tml* locus is unknown. If all three genes (*1*, *2* and *4*, the so-called *onc* genes) are lost, transformation still occurs but no tumours develop (Weiler

and Schröder, 1987). This indicates that although necessary for gall induction, phytohormones do not appear to play a rôle in the initial stages of infection.

It has been found that there is significant sequence homology between the *iaaM* gene (tryptophan monooxygenase) of *P. savastanoi* and that of *tms1* from *A. tumefaciens* and between the *iaaH* gene (IAAm hydrolase) of *P. savastanoi* and that of *tms2* from *A. tumefaciens* (Yamada *et al.*, 1985; Morris, 1986).

Plasmid-less strains of *A. tumefaciens* have been found to secrete IAA *in vitro*, although at a much reduced level. This has lead to the suggestion that there may be two operons for synthesis, one chromosomal and the other borne on the Ti-plasmid. It has been suggested that this production may have an influence on tumourigenesis (Liu and Kado, 1979). At the moment however, the situation remains unclear.

In addition to phytohormones, the cells transformed by *Agrobacterium* also produce an unusual group of compounds called opines which are not detected in untransformed plant tissue (Tempé and Goldman, 1982). These opines are used as sources of carbon, nitrogen and energy by the bacteria while the plant is unable to metabolise them. The genes for opine synthesis and degradation as well as the genes concerned with tumour formation are coded by the Ti-plasmid (Garfinkel and Nester, 1980). The increased cell numbers of these galls is clearly a means of ensuring that there are many more transformed cells serving the bacterium in the production of opines.

1.5.4 *Bradyrhizobium japonicum*

Sekine *et al.* (1988) detected the presence of IAAm, IAA and IAAm hydrolase activity in cultures of *Bradyrhizobium japonicum* which is a genus of slow-growing rhizobia (Jordan, 1982). From these results it was concluded that the pathway of IAA synthesis

from tryptophan to IAA in this species was, like *Pseudomonas savastanoi* (**Fig. 1.11.**) and *Agrobacterium tumefaciens* (**Fig. 1.12.**), via IAAm.

1.5.5. *Frankia* spp.

The production of IAA in non-leguminous root nodule symbioses has also been studied, although not as intensively as in the *Pseudomonas*, *Agrobacterium* or *Rhizobium*-legume associations. This is partly because the bacterial partner has only been successfully grown in pure culture relatively recently (Callaham *et al.*, 1978). The nodules of these actinorhizal plants are colonised by an actinomycetous bacterium of the genus *Frankia* of which there many different strains (Dixon and Wheeler, 1986). The microsymbiont nodulates plants over a wide taxonomic distribution and to date *Frankia*-nodulated plants have been found in twenty-five plant genera through eight plant families (Bond, 1976; Dixon and Wheeler, 1986).

As is the case in leguminous plants, root hair curling accompanies infection in actinorhizal plants where nodules develop as highly branched lateral roots (Angulo Carmona, 1974). It has been suggested that growth substances play a role in nodule formation as the levels of IAA, cytokinins and gibberellins are higher in infected than uninfected roots (Wheeler *et al.*, 1979) and in addition, lateral root primordia have been induced by the application of auxin, cytokinin and alpha-(2,4-dichlorophenoxy)acetic acid (2,4-D) (Angulo Carmona, 1974). Isopentenyl adenosine (iPA) has been identified as the single major cytokinin in the culture medium of *Frankia* sp. HFPArI3 (Stevens and Berry, 1988).

Wheeler *et al.* (1984) carried out an HPLC study of *Frankia* sp. AvcI1 culture filtrates after feeding with labelled tryptophan. They detected a radio-labelled peak with a retention time corresponding to that of IAA. They also found that the rate of IAA biosynthesis and turnover was slow in comparison to that found in *Rhizobium.* More

recently radiolabelled IAA, ILA, IEt and IM were detected by HPLC and combined GC-MS of *Frankia* sp. HFPArI3 culture filtrate after feeding with labelled tryptophan (Berry *et al.*, 1989). Despite this information the pathway of IAA biosynthesis in *Frankia* has not as yet been elucidated. However the presence of IEt, ILA and IM suggests it is likely to involve a tryptophan→IPyA→IAAld→IAA route (**Fig. 1.10.**) and as such resembles *Rhizobium* rather than *Agrobacterium* or *Pseudomonas* where tryptophan is converted to IAA via IAAm.

1.6. Genetic manipulation of *Rhizobium*

Rhizobium mutants capable of effective nodulation but ineffective in IAA biosynthesis have not as yet been identified (Bakanchikova and Lobanok, 1981). As a result, it has not been possible to determine with certainty whether or not IAA plays a rôle in the nodulation process. Testing the nodulation ability of *Rhizobium* strains unable to produce IAA, i.e. IAA-minus mutants, on plants, would enable this question to be addressed.

Transposon mutagenesis mediated via mobilising strains

Transposons are specific DNA segments with the ability to move as a unit in a more or less random fashion from one genetic locus to another (Berg and Berg, 1983). Transposon insertion may occur within a gene causing it to become non-functional and so in this way mutation is effected. On this principle, transposon Tn5 (**Fig. 1.13.**) has been frequently used for the random construction of auxotrophs. Introduction of the transposon from a donor to a suitable recipient, i.e. from *E. coli* into other gram-negative bacteria, and the subsequent insertion of the transposon into the recipient's genome has been previously demonstrated, for example in *Rhizobium leguminosarum* (Beringer *et al.*, 1978) and *Rhodobacter sphaeroides* (Hunter, C. N., 1988). The applicability of using transposon mutagenesis in non-enteric bacteria has been greatly facilitated by the construction of specific mobilising host strains of *E. coli* by Simon *et al.* (1983).

The transposon is borne on a plasmid upon which its transfer is dependent, and is maintained within the donor strain of *E. coli* through antibiotic selection. When these plasmid containing strains of bacteria, known as mobilising strains, are mixed with other gram-negative bacteria, the plasmid harbouring the transposon may be transferred, via a structure known as a pilus, from one cell to the other. This process is known as conjugal mating.

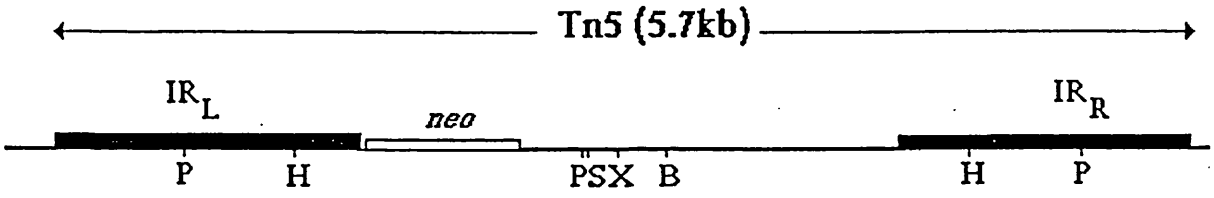


Fig. 1.13. Physical map of Tn5 showing restriction sites that can be used to identify the transposon after transposition; HindIII (H), PstI (P), SalI (S), XhoI (X) and BamHI (B). The phosphotransferase gene, *neo*, codes for resistance to the antibiotic neomycin. The inverted repeats (IR) at either side of the molecule [left and right] are responsible for the ability of the transposon to move from one genetic locus to another (Adapted from de Bruijn *et al.*, 1984).

When attempting to produce Tn5 mutants it is crucial that the vector plasmid is unable to replicate in the recipient bacterium. The development of 'suicide' plasmids that have only a limited replicative host range has solved this problem: these plasmids contain the ColE1 replicon which is functional only in *E. coli*, hence they are unable to replicate and are lost when introduced into the recipient cell. This acts as strong selective pressure for the transposon to integrate into the genome of the recipient. Simon *et al.* (1983) developed the pSUP range of plasmids [derived from pBR325] which can only replicate in *E. coli*. These are readily mobilisable and have a variety of antibiotic markers which allow for maintenance of the plasmid.

Selection of transconjugants i.e. cells which have acquired the carrier plasmid and should have the transposon integrated into the genome after mating, is accomplished because the transposon carries antibiotic resistance markers. Moreover as the mobilising *E. coli* strains are auxotrophic they are easily removed from recipient cells by plating on minimal medium. A schematic illustration of a di-parental mating is shown in **Fig. 1.14.**

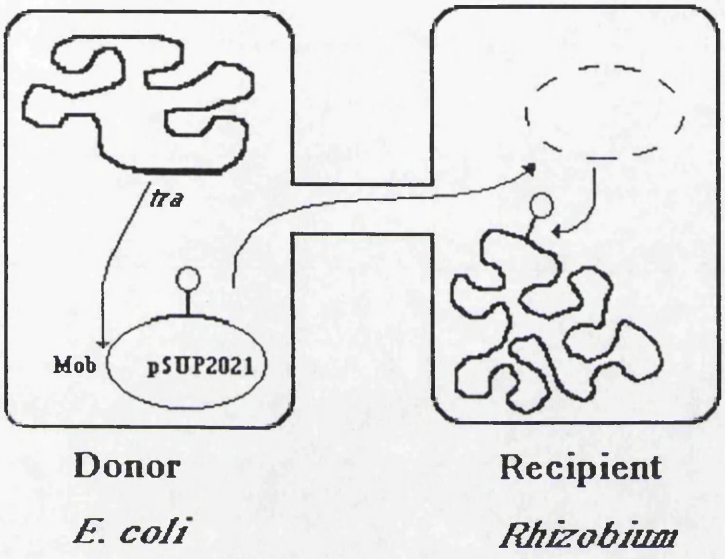


Fig. 1.14. Schematic representation of a di-parental mating between the 'suicide' plasmid donor, *E. coli* and the recipient cell, *Rhizobium*. Mobilising strains contain *trans*-acting transfer functions (*tra*) integrated on their genome which are responsible for e.g. cell-cell contact, pilus formation etc., and act on a site present on the plasmid (Mob) to initiate conjugal transfer. Once inside the recipient cell the vector plasmid is unable to replicate necessitating transposition into the genome and thereby bringing about mutagenesis (Adapted from Simon *et al.*, 1983).

Transposon mutagenesis was chosen over the use of chemical or U.V. light as the mutagenic agent. The disadvantages of mutagenic chemicals are that they are dangerous to handle and have frequent secondary mutations, for example, N-methyl-N-nitro-N-nitrosoguanidine (NG) is a potent mutagen but is also a carcinogen and has a high frequency of double mutations (Miller, 1972). When U.V. light is used as the mutagenic

agent, the rate of survival of bacteria is low (often only 0.1%) (Miller, 1972). Mutagenesis using the Tn5 transposon was chosen therefore, because it is safe, can be easily screened for, has a high frequency transposition rate and low insertional specificity and thus causes random mutation (Simon *et al.*, 1983).

1.7. Outline of the aims of the project

The aim of this project was to study the production of indolic compounds, particularly IAA, by *Rhizobium phaseoli* 8002 *in vitro* and in association with its host plant, *Phaseolus vulgaris* cv. Canadian Wonder. This species was chosen as it had been used previously in experiments with 8002 (Atzorn *et al.*, 1988) and in addition, is known to form nodules relatively easily with this strain. IAA has been postulated as playing an important physiological rôle in the development and/or maintenance of the mature nodule. It is essential therefore to expand our knowledge about the production of this compound by the bacterium in culture and in symbiosis.

1.7.1. The identification and quantification of indolic compounds which accumulate in tryptophan (+) and (-) medium of *Rhizobium phaseoli* 8002

The identity and quantity of indoles which accumulate in (+) and (-) tryptophan culture medium was investigated in order to determine whether there are paths of synthesis restricted to tryptophan-enriched cultures (Ernstsen *et al.*, 1987).

1.7.2. The effect of GA_3 on IAA accumulation in the culture medium of *R. phaseoli* 8002

Enhanced levels of IAA have been reported in dwarf pea seedlings sprayed with gibberellic acid (GA_3) (Law and Hamilton, 1984; Law, 1987). As they found additionally that exogenous D-tryptophan was more actively converted to IAA than L-tryptophan,

they postulated that D-tryptophan is the immediate precursor of IAA and that the racemisation of L- to D-tryptophan is GA controlled (Law and Hamilton, 1985). If this was the case D-tryptophan might act as a segregated pool of tryptophan available for IAA synthesis (Brian and Hemming, 1958). The model proposed to explain the roles of GA_3 and D-tryptophan in IAA biosynthesis is shown in **Fig. 1.15.** (Law and Hamilton, 1985).

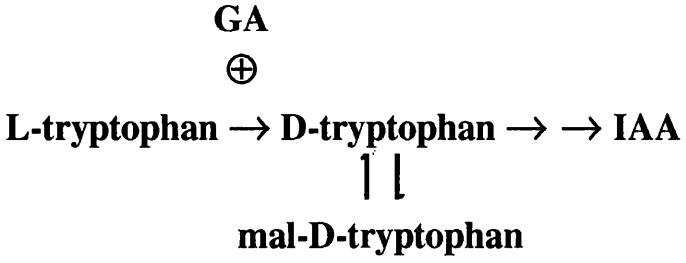


Fig. 1.15. The possible role of GA_3 and D-tryptophan in IAA biosynthesis (After Law and Hamilton, 1985; and Law, 1987). Malonyl-D-tryptophan (mal-D-tryptophan) is the conjugated form of D-tryptophan.

Rhizobium phaseoli and its host plant, *Phaseolus vulgaris* are both known to synthesise IAA and gibberellic acid (GA_3) *in vivo* (Atzorn *et al.*, 1988) and thus the possibility of synergism between these plant growth substances was investigated.

1.7.3. IAA metabolism by *Rhizobium phaseoli in vitro*

Rhizobium cultures were fed radiolabelled tryptophan and IAA in order to establish which metabolites were formed and from this, to elucidate the pathways of IAA synthesis and catabolism operating in the bacterium *in vitro.*

1.7.4. IAA metabolism by detached root and nodule tissue of *Phaseolus vulgaris*

The products of radiolabelled tryptophan and IAA feeds to detached root and nodule tissue of the host plant *Phaseolus vulgaris* were analysed by high performance liquid chromatography (HPLC) to investigate **(a)** differences in the metabolism between root

and nodule tissue and **(b)** to determine whether differences in pathways of IAA metabolism between bacterium and host plant might be used as 'markers' for IAA production by the bacterium in the symbiotic state.

1.7.5. The creation of IAA mutants

R. phaseoli mutants, created by transposon mutagenesis (**1.6.**), with IAA levels significantly less or greater than the wild-type strain, as judged by Salkowski reagent, were isolated prior to quantification by HPLC. IAA accumulation with time in the culture medium of the highest and the lowest IAA-producing mutant was investigated (0 to 96 h after flask inoculation).

1.7.6. The effect of low and high IAA-producing *Rhizobium* mutants on nodulation of *Phaseolus vulgaris*

Reports on the relationship between rhizobial IAA production and infection/nodulation have been contradictory. Hunter, W. J. (1988) found 'super-nodulation' by high IAA-producers of *Bradyrhizobium japonicum.* Other authors however, have found no correlation between IAA production by *Rhizobium* and the ability to infect/nodulate the leguminous host plant (Badenoch-Jones *et al.*, 1983; Wang *et al.*, 1982; Atzorn *et al.*, 1988). In order to clarify whether such a correlation exists, an attempt was made to create mutants with altered IAA levels (**1.7.5.**), and possibly an IAA-minus mutant of *R. phaseoli* 8002 by transposon mutagenesis (**1.6.**).

CHAPTER 2

MATERIALS AND METHODS

2.1. General microbiological techniques

2.1.1. Maintenance of *Rhizobium phaseoli* strains

Rhizobium phaseoli strains 8002, RW4 and 4292 (Dr A. W. B. Johnston, John Innes Institute, Norwich) were maintained on yeast mannitol agar slopes (**Appendix A**). These slopes were subcultured frequently and regular checks were carried out to ensure that they were contaminant-free. In addition, *Rhizobium,* wild-type and mutant stocks were mixed with sterile glycerol (15%) and stored at -70°C.

2.1.2. Aseptic techniques

In order to limit the risk of microbial contamination, all growth media and flasks were autoclaved at 120°C for 20 min at 1.1 kg/cm^2. The inoculation of flasks, preparation of plates, removal of aliquots of culture and addition of radioactive labels through a filter sterilisation unit (Sartorius, Minisort NML 0.2 μm, Gottingen, Germany) were carried out in a laminar flow-hood. Flasks containing media were left for approximately four days to ensure sterility before the addition of inoculum.

2.1.3. Estimation of cell numbers

a) Estimates of cell numbers were made spectrophotometrically. An aliquot of culture was removed from the flask, diluted as necessary and the optical density at 500 nm was measured in a 1 cm cell (Vincent, 1970). Uninoculated growth medium was used as a blank to calibrate the machine. The resulting optical density was compared with a calibration curve of optical density versus cell number per ml.

b) Viable cell counts were estimated by removing an aliquot of culture, preparing a serial dilution and then plating onto agar plates. After 2/3 days, the colonies were large enough to be counted. The addition of Congo Red (**Appendix A**) to the plates was used to check for the presence of contaminating bacteria. In contrast to many bacteria,

Rhizobium absorbs very little of this dye and so remain practically colourless (Hahn, 1966; Vincent, 1970).

2.2. Culture of *Rhizobium in vitro* and extraction of indolic compounds from the culture medium

2.2.1. Growth conditions of *Rhizobium*

Variations on the growth conditions, including the amount of L-tryptophan added to the culture medium, of individual experiments are detailed in **2.2.3.**

Rhizobium inoculum, grown on yeast mannitol slopes, was cultured in 100 ml defined liquid medium (modified from Vincent, 1970, **Appendix A**) in 250 ml conical flasks. The cells were shaken on an orbital shaker (Scotlab VSL) at 120 rpm in darkness at 27°C. Once the cultures had reached the desired optical density, as measured by absorbance at 500 nm (Pye Unicam, SP8-500 UV/VIS Spectrophotometer), an aliquot was removed aseptically to determine accurately the number of cells per ml of culture extracted. The culture was plated, at dilutions between 10^{-3} and 10^{-8}, onto agar plates containing the dye Congo Red to permit detection of contaminants.

2.2.2. Extraction of indolic compounds from *Rhizobium* culture medium

Sodium diethyldithiocarbamate (20 mM) was added to the culture medium as an antioxidant (Ernstsen *et al.*, 1986) prior to centrifugation (MSE Mistral 2L, Sussex, U.K.) at 2000 g for 30 min. The supernatant was gently poured off and a [2'-^{14}C]-IAA internal standard (specific activity 2.0 GBq mmol^{-1}, Amersham International, Amersham, U.K.) was added (5 MBq per 100 ml). The medium was then adjusted to pH 8.0 and 50 ml K_2HPO_4 buffer (0.1 M, pH 8.0, adjusted with KH_2PO_4, **Appendix C**) was added to each flask. Phenolic compounds and other impurities were removed by the addition of

insoluble polyvinylpolypyrrolidone (PVP, Sigma Chemical Co.) (50 mg ml^{-1}) (Glen *et al.*, 1972) and stirring for 10 min before removal by filtration.

Partitioning was then carried out as outlined in **Fig. 2.1.** (Sandberg *et al.*, 1987). The buffered supernatant (pH 8.0) was partitioned twice against 1/2 volume of distilled ethyl acetate to give a neutral/basic ethyl acetate fraction. The pH of the aqueous phase was then adjusted to 3.0 using concentrated sulphuric acid. This was then partitioned against 3 x 1/3 volume of ethyl acetate resulting in an acidic ethyl acetate fraction. Water was removed from the acidic and the neutral/basic ethyl acetate fractions by the addition of anhydrous Na_2SO_4. This was stirred for 10 min and filtered before evaporating to dryness *in vacuo* at <40°C (Buchi Rotary Evaporator). The residue, redissolved in 50:50 HPLC-grade ethyl acetate and methanol (Rathburn Chemicals Ltd. Walkerburn, Scotland), was transferred to a vial before drying down under O_2-free nitrogen (British Oxygen Company). The sample was dissolved in 1 ml HPLC-grade methanol and a 10 µl aliquot was removed to determine the radioactivity of the sample. The aliquot was added to 2 ml of Ecoscint liquid scintillation fluid (National Diagnostics, South Somerville, N. Jersey) and the radioactivity determined by liquid scintillation counting (LKB 1211). The sample was then analysed by reversed-phase HPLC (**2.5.**)

2.2.3. Indole accumulation in the culture medium of *R. phaseoli* 8002

Rhizobium phaseoli strain 8002 (wild-type unless otherwise stated) was used for each of the following investigations into indole accumulation in the culture medium. In each case the medium was cultured and indolic compounds extracted as detailed in **2.2.1.** and **2.2.2.**

a) Identification and quantification of the indoles produced by *Rhizobium phaseoli* 8002 in tryptophan (+) and (-) medium

Cells were grown in defined liquid medium (+) (200 mg l^{-1}) or (-) L-tryptophan.

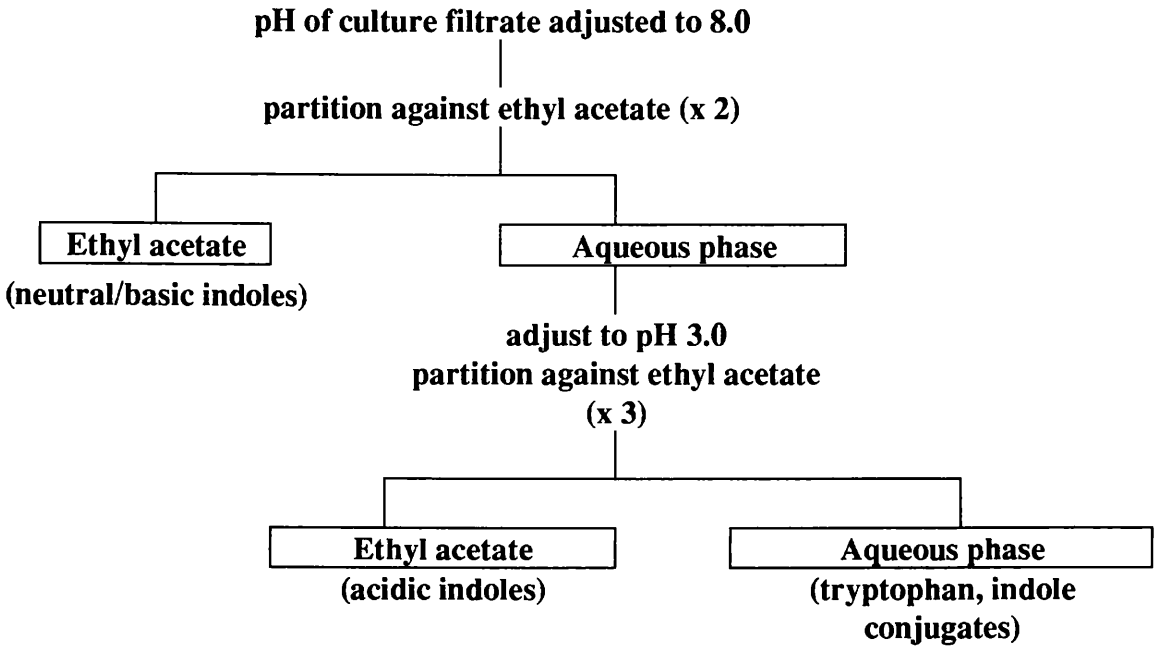


Fig. 2.1. Partitioning procedure for the separation of indoles, extracted from the culture medium of *Rhizobium phaseoli* 8002, into neutral/basic, acidic and conjugate fractions (Adapted from Sandberg *et al.*, 1987).

b) The effect of gibberellic acid (GA_3) on IAA accumulation

Gibberellic acid (GA_3)(Sigma) was filter sterilised into autoclaved defined liquid medium to give final concentrations of 10^{-5} and 10^{-6} M GA_3.

c) IAA accumulation by Tn5 mutants of *Rhizobium*

Mutant cells were grown in L-tryptophan (+) medium (200 mg l^{-1}) containing neomycin (30 μg ml^{-1}) (**Appendix D**).

d) Time course of IAA accumulation by Tn5 mutants of *Rhizobium*

The highest and the lowest IAA-producing mutants were grown in L-tryptophan (+) liquid medium (200 mg l^{-1}) containing neomycin (30 μg ml^{-1}) and harvested at various times between 0 and 96 h after inoculation.

e) IAA accumulation by mutant and wild-type *Rhizobium* after re-isolation from *Phaseolus vulgaris* nodules

The accumulation of IAA by re-isolated low and high IAA-producing mutants and wild-type 8002 was investigated. Cells were grown in culture medium supplemented with L-tryptophan (200 mg l^{-1}).

2.2.4. Metabolism of [^{3}H]-tryptophan and [^{14}C]-IAA by cultures of *Rhizobium phaseoli* 8002

Radiolabelled compounds

L-[5-^{3}H]-tryptophan (specific activity 925 GBq $mmol^{-1}$) and [2'-^{14}C]-IAA (specific activity 2 GBq $mmol^{-1}$) were both purchased from Amersham International, Amersham, U.K. These radiolabels will be referred to in this thesis as [^{3}H]-tryptophan and [^{14}C]-IAA.

a) [^{3}H]-Tryptophan feed to *Rhizobium*

Cells, grown in medium supplemented with L-tryptophan (5 mg l^{-1}) were fed [^{3}H]-tryptophan (50 MBq), incubated for 3 h in an orbital shaker culture (120 rpm) at 27°C and extracted immediately as detailed in **2.2.2.**

b) [^{14}C]-IAA feed to *Rhizobium*

Cells, grown in medium supplemented with L-tryptophan (5 mg l^{-1}) were fed [^{14}C]-IAA (25 MBq), incubated for 3 h in an orbital shaker (120 rpm) at 27°C in darkness and extracted immediately as detailed in **2.2.2.**

2.3. Experiments concerned with *Phaseolus vulgaris*

2.3.1. Growth conditions of *P. vulgaris*

Seeds of *Phaseolus vulgaris* cv. Canadian Wonder were germinated in trays of Vermiculite and Perlite (50:50). Germinated seedlings were transferred into individual pots containing Vermiculite, Perlite and John Innes compost (45:45:10). Additional nutrients (Crones-N salts, 0.2 g l^{-1} and 0.5 ml l^{-1} of A-Z nutrients [Bond and Wheeler, 1980]) were watered into the pots initially and after 5 weeks, if necessary. The composition of the Crones and the A-Z nutrient solution is shown in **Appendix B**. The pH of the growth medium plus nutrients was 6.5 in distilled water (50:50). Pots were sterilised initially by swabbing thoroughly with methanol before addition of the growth medium.

The plants were watered with tap water as necessary and grown in a controlled environment growth room (16 h light, 23°C; 8 h dark, 19°C). Light was provided by Osram 75/85 Warm White fluorescent tubes.

2.3.2. Inoculation of *Phaseolus* with *Rhizobium*

Rhizobium was grown on defined minimal medium (**Appendix A**) plates at 28°C. For mutant bacteria, neomycin (30 μg ml^{-1}) was added to the plates. When thick colonies had formed, the cells were scraped off, suspended in distilled water and then centrifuged, if necessary, to concentrate the cells. Aqueous suspensions were used to inoculate 7-day-old seedlings which were then grown on for 10 weeks. Approximately equal numbers of bacteria (2.5 x 10^{10} cells) were used to inoculate each plant.

When wild-type and mutant bacteria were inoculated onto plants, precautions were taken to avoid strain cross-infection by careful watering of the plants and by keeping plants inoculated with different strains separate in the growth-room.

2.3.3. Re-isolation of *Rhizobium* from *Phaseolus* nodules

Nodules were removed from the root and surface sterilised by shaking in ethanol for 15 seconds. The ethanol was poured off and replaced with sterile distilled water. After washing twice more to ensure the removal of all the alcohol, the nodules were ground in a glass homogeniser containing sterile distilled water. From this, a serial dilution series was prepared prior to plating onto defined minimal medium agar containing Congo Red (**2.2.1.**)(**Appendix A**). After colony formation, the isolates were subcultured on Congo Red until free from contaminants.

2.3.4. Neomycin resistance of re-isolated mutant cells

Bacteria, re-isolated from the plants inoculated with mutant cells, were grown on minimal medium plates (+) and (-) neomycin (30 μg ml^{-1}) to determine antibiotic resistance. All plates additionally contained Congo Red to check for contamination.

2.3.5. Determination of nitrogenase activity using the acetylene reduction assay

The observations of Minchin *et al.* (1983) were taken into consideration; however, the assay was considered adequate for comparisons of nodular nitrogenase activity as required by this study.

Freshly-harvested roots of *Phaseolus vulgaris* cv. Canadian Wonder, nodulated with *R. phaseoli* 8002, wild-type and low and high IAA-producing mutants, were assayed for nitrogenase activity using the acetylene reduction technique (Hardy *et al.*, 1973; Turner and Gibson, 1980).

The whole root of each plant was placed in an incubation jar (volume 550 ml) and sealed with a Suba-Seal vaccine stopper (Freeman Limited). Air (50 ml) was removed with a syringe fitted with a needle and replaced with 50 ml of acetylene gas, giving a concentration of approximately 10%. The acetylene was well mixed using the syringe and the jars incubated in the dark for 1 h at 25°C. At the end of the incubation period the gas was mixed again and 0.5 ml samples of gas withdrawn.

The ethene produced was assayed using a Pye 104 gas chromatograph operated isothermally at 60°C with a 1 m x 1/4 inch steel column of Poropak N (100-120 mesh), a carrier gas of oxygen-free nitrogen (flow rate 35 ml min^{-1}) and a flame ionisation detector (hydrogen/air flame). The relationship between peak height and concentration was linear over the range used and quantification of detector response was by comparison with the peak height given by a standard (103.6 ppm ethene in argon, British Oxygen Company Special Gases).

2.3.6. Dry weight of plant tissue

On completion of the acetylene reduction assay, the plants were divided into roots, shoots and nodules, put into paper bags and dried to constant weight at 70°C.

2.4. Metabolism of tryptophan and IAA by *Phaseolus* roots and nodules

2.4.1. Incubation conditions

Plants were grown and inoculated with *Rhizobium* as described in **2.3.1.** and **2.3.2.**, then the lower 2 cm of the roots and the nodules were harvested. Before incubation, the nodules were cut in half to facilitate diffusion of the label into the tissue. The incubation conditions of roots and nodules with [2'-^{14}C]-IAA (specific activity 2 GBq mmol^{-1}) and [5-^{3}H]-tryptophan (specific activity 925 GBq mmol^{-1}) are shown on **Table 2.1.** All feeds were carried out in flasks in the dark at 25°C in 25 ml KH_2PO_4 buffer (1 mM) adjusted to pH 7.0 with NaOH.

2.4.2. Extraction of indolic compounds from root and nodule material

After incubation, sodium diethyldithiocarbamate as an antioxidant, (Ernstsen *et al.*, 1986) and distilled methanol (50 and 20 ml to roots and nodules respectively) were added to the flasks. The tissue was ground in a mortar and left for 1 h at room temperature in the dark. PVP (50 mg ml^{-1}) was added, stirred for 10 min and the liquid filtered through an alpha-floc pad (**Appendix C**) under vacuum. The methanolic extract was evaporated to dryness *in vacuo* on a Buchi rotary evaporator. The residue was re-dissolved in HPLC-grade methanol and ethyl acetate (50:50) and transferred to a vial before drying under oxygen-free nitrogen. HPLC-grade methanol (1 ml) was added, a 10 µl aliquot was added to 2 ml of Ecoscint liquid scintillation fluid (National Diagnostics, South Somerville, N. Jersey) and the radioactivity in each fraction determined by liquid scintillation counting (LKB 1211). The sample was then analysed by reversed-phase HPLC (**2.5.**).

plant tissue	weight (g)	radiolabel added	incubation medium	incubation time
root (x 2)	6 g	[^{14}C]-IAA (83.3 MBq)	distilled water	6 h
root (x 2)	6 g	[^{3}H]-tryp. (333 GBq)	distilled water	6 h
root	2g	[^{3}H]-tryp. (333 GBq)	PO_4 buffer	12 h
root	2 g	[^{14}C]-IAA (41.6 GBq)	PO_4 buffer	12 h
nodule	1.5 g	[^{14}C]-IAA (83.3 MBq)	distilled water	6 h
nodule	1 g	[^{14}C]-IAA (41.6 GBq)	PO_4 buffer	12 h
nodule	1 g	[^{3}H]-tryp. (333 GBq)	PO_4 buffer	12 h
nodule	1 g	[^{14}C]-IAA (41.6 GBq)	PO_4 buffer	24 h

Table 2.1. Summary of the incubation conditions of detached root and nodule tissue of *Phaseolus vulgaris* with [^{3}H]-tryptophan and [^{14}C]-IAA. The fresh weight of tissue (g), radioactivity added, incubation medium and length of incubation time of radiolabelled feeds. Phosphate buffer was 1 mM KH_2PO_4 adjusted to pH 7.0 with NaOH. All feeds were carried out in darkness at 25°C.

2.5. Analysis of indolic compounds by high performance liquid chromatography (HPLC)

2.5.1. Chromatography

Solvents were delivered at a flow rate of 1 ml min^{-1} by a Spectra Physics SP 8700 liquid chromatograph (San Jose, Cal., USA). Samples were introduced via a Valco sample valve with a 250 µl sample loop. For reversed-phase separation a 250 x 5 mm i.d. steel column with a 5 µm reversed-phase support (ODS Hypersil, Shandon Southern Products, Runcorn, Cheshire, UK.) was eluted **(a)** over 30 min with a gradient of 30-70% or **(b)** 0-10 min 5% followed by 10-30 5-50% methanol in aqueous buffer. Acidic extracts were analysed in an acidic buffer (1% acetic acid, v/v) and neutral and basic indoles were analysed in 0.5% acetic acid (v/v) adjusted to pH 6.5 with NH_4OH. Normal phase separations were carried out isocratically on a 250 x 5 mm i.d. 5 µm Spherisorb CN Nitrile column using a mobile phase of *n*-hexane in ethyl acetate containing 0.5% acetic acid (v/v). 1% ethanol (v/v) replaced 0.5% acetic acid when neutral and basic samples were analysed.

2.5.2. Detection of indolic compounds

a)Fluorescence monitor

Fluorescence offers a highly selective and sensitive method of indole detection. Crozier *et al.* (1980) reported that the limit of detection for reversed-phase HPLC was 1 pg, and for normal phase, 50 pg. Most indoles can be detected with the excitation wavelength set at 280 nm and emission at 350 nm. There are however two notable exceptions to this, IAAld and IAld do not fluoresce and therefore cannot be detected in this way (Burnett and Audus, 1964). In addition, it was found that although ILA fluoresces in reversed-phase solvents (methanol and water), it does not fluoresce in normal phase solvents (ethyl acetate and hexane). For each of these compounds, a U.V. absorbance monitor set at 280 nm was used instead for detection (**2.5.2b.**).

In the current investigation, HPLC eluant was monitored with a Perkin-Elmer 650-10S spectrofluorimeter operating at 280 nm (excitation) and 350 nm (emission) and fitted with a 16 µl flow cell.

b) Ultra violet monitor

Ultra violet (U.V.) absorbance is not as sensitive or as selective as fluorescence as a means of detection. U.V. however, is valuable for the detection of those indoles which are not fluorescent. In this project, HPLC eluant was monitored with a U.V. monitor (Pye-Unicam LC 871 UV-VIS) operating at 280 nm.

c) Radioactivity monitor

When analysing the labelled products of metabolic studies, the HPLC eluate was directed to a continuous-flow liquid scintillation monitor operating in the homogenous counting mode (Reeve and Crozier, 1977). In this type of monitor, the column eluate is mixed with a liquid scintillation cocktail (**Appendix C**) pumped by a reagent delivery unit (Reeve Analytical, Glasgow, UK) at 3 ml min^{-1} for reversed-phase, and 2 ml min^{-1} for normal-phase chromatography. The mixture passes through a spiral glass flow-cell (500 µl volume) positioned between the photomultiplier tubes of the radioactivity monitor (Reeve Analytical, Glasgow, UK).

2.5.3. Authentic indole standards

The following authentic indolic standards, dissolved in HPLC-grade methanol, were stored in amber glass vials in the freezer at -20°C: IAA, IAAsp, IAAm, IAld, IAAld, IAN, IBA, ICA, IEt, ILA, IM, IPyA, IPA, OxIAA, indole acetone, indole, tryptophan and tryptamine. These compounds were all purchased from the Sigma Chemical Company (Poole, Dorset) with the exception of OxIAA and IM which were received as gifts.

2.5.4. Quantitative analysis of IAA production by isotopic dilution

Because of the requirement for purification and chromatographic procedures, sample losses inevitably occur during analysis of endogenous indoles which affect adversely the accuracy of quantitative estimates. However, such errors can be corrected when carrying out an isotopic dilution analysis as described by Rittenberg and Foster (1940). This involves the addition of a known amount of internal standard to the sample at the extraction stage. The most suitable internal standard is an isotopically-labelled analogue of the compound of interest, which can be differentiated from the endogenous component by analytical procedures, but otherwise tends to behave in an identical manner. As a consequence, once the internal standard is added to the extract, the isotope/endogenous substrate ratio is maintained, irrespective of sample losses encountered during purification. The amount of endogenous compound extracted from the culture medium (**Y**) can, thus, be calculated from the isotopic dilution equation

$$\mathbf{Y = ([Ci / Cf] - 1) X}$$

where **X** = the amount of internal standard added to the sample, **Ci** = initial specific activity of the internal standard and **Cf** = final specific activity of the internal standard after dilution with endogenous IAA.

During these investigations, [^{14}C]-IAA was used as the internal standard. After extraction and partitioning, 1 x 10^4 dpm of each sample was injected onto the HPLC. The fluorescent IAA peak was both quantified by reference to a 1-100 ng IAA standard curve and collected for determination of radioactivity by liquid scintillation counting (eluant:Ecoscint, 1:4) (LKB 1211).

2.6. Mass spectrometry

An MS12 mass spectrometer (Kratos) with a direct insertion probe was used to obtain positive ion 70 eV impact mass spectra of authentic IAA, ILA, IEt, IM and IAld (Sigma) and putative IAA, ILA, IEt and IM samples from the culture medium of *R. phaseoli* 8002. Samples were purified sequentially by reversed-phase and normal phase HPLC prior to MS analysis. The standards and sample peaks from HPLC were dissolved in methanol and analysed at 165°C (IAld), 170°C (ILA, IM), 180°C (IEt) and 190°C (IAA). Analyses were kindly carried out by Mr. A. Ritchie, Department of Chemistry, University of Glasgow.

2.7. Mutagenesis of *Rhizobium phaseoli* 8002 using transposon Tn 5

Using the plasmid-borne transposon, Tn5 (Simon *et al.*, 1983) (**Fig. 1.11.** and **1.12.**) to introduce random mutations into the genome of *R. phaseoli* 8002 wild-type, an attempt was made to create non, high or low IAA-producing mutants (Beringer *et al.*, 1978; de Bruijn and Lupski, 1984). These were then be tested on *Phaseolus* plants in order to determine whether bacterial IAA production is involved in the initial infection processes and/or development of the nodule.

2.7.1. Donor bacteria

Plasmid pSUP2021 which carries transposon Tn5 was maintained within *E. coli* strain 17-1[2021] (Simon *et al.*, 1983). Forty-eight hours prior to mating, cells were streaked onto an LB-broth (**Appendix A**) plate containing neomycin (Nm) and ampicillin, 30 µg ml^{-1} and 50 µg ml^{-1} (**Appendix D**) respectively and incubated at 37°C. These antibiotics are necessary for the maintenance of transposon and plasmid respectively. At the end of this period, the cells were resuspended gently in 0.5 ml LB-broth. Cell numbers per ml were calculated by measuring the optical density of the culture medium at 600 nm. For

this strain of *E. coli*, previous counts had determined that an O.D. of 1 cm^{-1} was equal to approximately 8 x 10^8 cells ml^{-1} (A. T. Gardiner, University of Glasgow, personal communication).

2.7.2. Recipient bacteria

R. phaseoli 8002 wild-type was grown in defined minimal liquid medium shaking (120 rpm) at 27°C for 3 days. The absorbance of the culture at 500 nm was compared with a standard growth curve from which the number of cells per ml was calculated.

2.7.3. Determination of rifampicin sensitivity in *R. phaseoli* 4292

Rifampicin is an antibiotic which binds to the β-subunit of RNA polymerase preventing RNA synthesis (Maniatis *et al.*, 1982). A rifampicin resistant strain of *R. phaseoli* (strain 4292) was plated onto the following concentrations of rifampicin in minimal media (**Appendix D**): 0, 0.2, 2, 20 and 200 μg ml^{-1}. However as this strain showed no resistance to the antibiotic at these concentrations, this was repeated with 0.2-200 ng ml^{-1} rifampicin.

2.7.4. Determination of neomycin sensitivity in *R. phaseoli* 8002

Neomycin (Nm) binds to the ribosomal components of the bacteria thus inhibiting protein synthesis (Maniatis *et al.*, 1982). *R. phaseoli* 8002 at dilutions from 10^{-3} to 10^{-8} were plated in duplicate onto concentrations of Nm between 0 and 50 μg ml^{-1} and incubated at 26°C for five days. The colonies were counted and used to construct a neomycin killing curve.

2.7.5. Mating

After calculating the number of bacteria per ml, the cells were mated at the desired ratios. For mating to be successful, it was important that there were more recipient bacteria than *E. coli*, which because of its very fast doubling rate, would otherwise

quickly out-compete the recipient cells.

Rhizobium/E. coli mixture (100 µl) was prepared and 50 µl of this pipetted onto a well-dried L-broth plate and incubated (6 h, 37°C). After incubation the *Rhizobium/E. coli* mixture was scraped off and resuspended in minimal media (100 µl) and a dilution series prepared (10^0-10^{-3}). The bacteria were plated on minimal medium plates containing Nm (30 µg ml^{-1}) to select for transconjugants, additionally the medium contained 200 mg l^{-1} L-tryptophan to screen for IAA production (medium modified from Bakanchikova and Lobanok, 1981; **Appendix A**). Plates were spread, half with mated bacteria and half with unmated as a control. In addition, unmated *Rhizobium* (diluted 10^0-10^{-3}) were plated onto controls **(a)** Nm (+) to show that the Nm was lethal and **(b)** Nm (-) plates to ensure that the culture itself was viable. Plates were left to incubate at 26°C for 7 days.

As 17-1[2021] is a histidine auxotroph it is thus selected against on minimal medium. In addition, *Rhizobium* is known to be sensitive to neomycin at this concentration and therefore only cells which have the transposon (and therefore the antibiotic resistance gene) are able to form colonies.

2.8. Screening of mutant colonies for IAA

At the end of the incubation period the transconjugant colonies were tested for IAA production with Salkowski reagent (as modified by Gordon and Weber, 1951). This reagent (0.5 M $FeCl_3$ in 35% perchloric acid, 1:50) changes from colourless to pink in the presence of IAA.

2.8.1. Reaction of IAA with Salkowski reagent

The following amounts of IAA: 0, 5, 10, 25, 50, 100, 250, 500 and 1000 ng were added to 100 µl of Salkowski reagent and left for 20 min for the colour change to develop. The

colour produced in the reaction between authentic IAA and Salkowski reagent was found to be proportional to the amount of IAA present.

2.8.2. Salkowski testing

The original method of testing *Rhizobium* colonies with Salkowski reagent (Bakanchikova and Lobanok, 1981) involved duplicate plating of the colonies prior to screening by spotting with Salkowski reagent. A variety of methods for testing the *Rhizobium* colonies were investigated, however the following procedure was found to be the most efficient.

A bent inoculating loop was used to slice down into the centre of the colony and the underlying agar. The loop was then twisted and lifted removing the piece of agar. This method solved the problem of maintaining the sterility of the plate while removing the agar plug. Additionally, because the strong acidity of the Salkowski reagent renders the colonies rapidly non-viable, the original method necessitated duplicate plating. However, using the above technique, where the reagent does not come into contact with the colony, there was no need to do this.

Using the above method, the volume of the agar removed and tested with the reagent was calculated thus: $\pi r^2 h = \pi \times 2^2 \times 5.5 = 69$ mm^3 = 69 µl, where the diameter of the loop was 4 mm and the depth of the agar, 5.5 mm. Once removed, the agar plugs were placed in 0.5 ml Eppendorf tubes containing 20 µl of Salkowski reagent, mixed and left for 15 min, during which time the agar completely dissolved and the colour developed fully. The tubes, against a background of white paper, were given an arbitrary score from 5 to 0 with 5 being the darkest shade of pink and 0 being colourless. Those which gave the brightest or the palest reactions were transferred onto fresh minimal medium plates containing tryptophan (200 mg l^{-1}) and Nm (30 µg ml^{-1}) as before and incubated at 27°C until the colonies were large enough to be re-tested.

After the second round of testing was completed, those with scores of 0, 1 and 5 were chosen for further examination. The colonies of interest were re-plated onto Nm 30 plates containing Congo Red to ensure that they were free from contamination (Hahn, 1966, Vincent, 1970) before being transferred into liquid culture.

2.8.3. Improved technique for screening for mutants with altered levels of IAA

Although the technique used in this thesis for the detection of mutants with increased/decreased IAA accumulation (modified from Bakanchikova and Lobanok, 1981) was satisfactory for the relatively small number of colonies created, testing of larger number of colonies would be time consuming. Since carrying out this work however, a new procedure has been developed by Bric *et al.* (1991) working with *Pseudomonas syringae* subsp. *savastanoi* and *Erwinia herbicola.* They developed the screening technique further to permit the rapid and accurate screening of large numbers of colonies. These authors inoculated plates of tryptophan-supplemented medium with the isolates, overlaid the medium with a nitrocellulose membrane and then incubated them until the colonies reached 1-2 mm in diameter. The membrane was removed to a filter paper saturated with Salkowski reagent and incubated until red haloes formed around the colonies. This procedure is a sensitive and specific assay for the qualitative determination of IAA production and avoids the need for replica plating (Bakanchikova and Lobanok, 1981) and the individual testing of each mutant as carried out in this thesis.

2.9. DNA hybridisation

In order to show that the transposon was present in the DNA of the mutants, it was extracted, digested with restriction endonucleases and separated using gel electrophoresis. The DNA was then transferred by Southern blotting to a nylon filter ready to be hybridised to a probe. The probe was prepared by digesting the plasmid on

which it is carried, separating the fragments, isolating the band of interest and labelling it with ^{32}P. If the DNA sequence of the probe is homologous to the DNA on the filter, it will hybridise to it. The positions of any hybridised pieces of DNA (and hence of the transposon) are then revealed by autoradiography. A flowchart showing the steps of hybridisation of the transposon DNA probe to the genomic DNA of *Rhizobium* is shown in **Fig. 2.2.**

2.9.1. Preparation of genomic DNA from *R. phaseoli*

Using the protocol detailed below (Dr C. N. Hunter, University of Sheffield, personal communication) genomic DNA was extracted from the cells of wild-type and mutant *Rhizobium.* Bacteria were grown as before in liquid culture (defined minimal media) until a dense culture had formed. Two sterile centrifuge tubes each containing 20 ml of culture were harvested at 13000 g for 1 hour at 4°C. After centrifugation, the tubes were placed on ice, the pellets resuspended in 10 ml of ice-cold membrane buffer (TE + 0.1 M NaCl) (**Appendix D**), vortexed and centrifuged at 9000 g for 10 min at 4°C. After washing twice more, the pellets were combined in one tube containing 10 ml ice-cold membrane buffer. 2 ml of a 10 mg ml^{-1} solution of lysozyme (**Appendix D**), freshly made up in membrane buffer, was added, mixed gently by rolling and left at 37°C for 10 min. After this time SDS (1%) and RNAase (20 µg ml^{-1}) (**Appendix D**) were added and left to incubate for 1 h at 37°C.

After incubation, proteinase K (**Appendix D**) was added (50 µg ml^{-1}) and the tube left to incubate at 37°C overnight. At the end of this period, an equal volume of phenol/chloroform (**Appendix D**) was added and the tube rolled very gently (1 h, 25°C) on an automatic roller (Luckham Multimix MM1) to avoid shearing the DNA. The tube was centrifuged (1 h, 2000 g) and the upper aqueous phase was removed carefully using a sterile wide bore pipette (as a further precaution against damaging the DNA) and placed in a new tube. This was repeated until all the interface (i.e. denatured protein) had

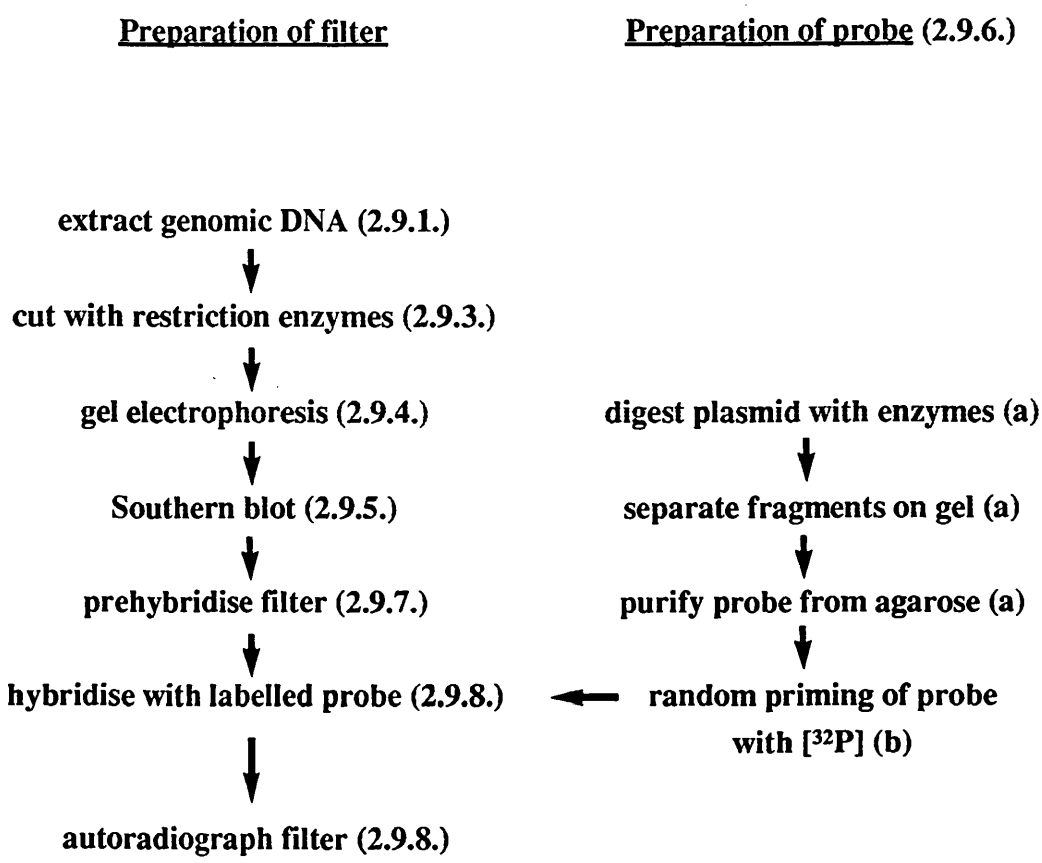


Fig. 2.2 Procedure for DNA hybridisation

Flowchart showing the sequential steps for hybridisation of genomic DNA to a [^{32}P]-labelled probe of transposon Tn5. Each of the steps is described in detail in the text (2.9.).

been removed. The DNA was then extracted as before using chloroform. The DNA-containing phase was removed to a fresh tube, 1/10 vol. Na acetate (3 M, pH 5.2) plus 2.5 vols. of ice-cold ethanol were added and the vial placed at -20°C for 2 hours. The DNA precipitate formed, was removed by spooling it around a hooked Pasteur pipette before being placed in a bijoux vial. The precipitate was washed with 2 x 1 ml of 70% ethanol, the last traces of which were removed by drying *in vacuo*. TE buffer (1x, 1 ml) (**Appendix D**) was added and the DNA left at R.T. to dissolve into the buffer.

2.9.2. Spectrophotometric determination of DNA

To determine the purity of a given sample of DNA, the absorbance of the solution was measured spectrophotometrically at 260 and 280 nm (Maniatis *et al.*, 1982). The following standard formula was used to calculate the concentration of DNA (µg ml^{-1}) of the solution:

(O.D. 260 - O.D. 280) x 2= x O.D.

An optical density of 1 corresponds to approximately 50 µg ml^{-1} for double-stranded DNA.

2.9.3. Cutting genomic DNA with restriction enzymes

Restriction enzyme digests were carried out using sterile reagents, pipette tips and tubes and gloves were worn throughout. These precautions are to guard against contamination with DNA-degrading enzymes.

For the digest, the following solutions were added in order to an Eppendorf tube: Analar H_2O (BDH Ltd., Poole, England) to make up total volume (30 µl), restriction enzyme buffer (1x), 1 µg DNA and 10 units μl^{-1} restriction enzyme (BDH Ltd., Poole, England).

The tube was incubated at 37°C overnight, after which the tube was pulse centrifuged. Dye stop (1x) (**Appendix D**) was added and the enzyme denatured by heating at 65°C for 5 min. The tube was pulse centrifuged again before loading onto the agarose gel.

2.9.4. Agarose gel electrophoresis

(a) Preparation of gel (Maniatis *et al.*, 1982)

The gel (1% agarose in 1x TAE, **Appendix D**) was prepared by heating until the agarose had completely dissolved and pouring into a gel-forming tray. When solid the comb was removed and the gel placed in a gel tank filled with 1 litre of TAE (1x) buffer, ensuring that no bubbles were trapped underneath.

(b) Electrophoresis (Southern, 1980)

Electrophoresis was left to proceed for 12 hours at 30 millivolts until the dye front had progressed 2/3 way down the gel.

(c) Gel staining

The gel was carefully transferred into a 0.5 μg ml^{-1} solution of ethidium bromide (**Appendix D**) for 1 h. Ethidium bromide is a fluorescent dye containing a planar group which intercalates between the stacked bases of the DNA. The fixed position of this group and its close proximity to the bases, causes dye bound to DNA to display an increased fluorescent yield compared to dye free in solution (Maniatis *et al.*, 1982).

After staining, the gel was destained for several hours using 1/2 hourly changes of distilled water. Once destaining was complete, the DNA was visualised by examination under a standard U.V. transilluminator (UVP, inc., San Gabriel, California) at 254 nm.

2.9.5. Southern blotting

In a Southern blot (Southern, 1975) DNA fragments are separated according to size by agarose gel electrophoresis, denatured, transferred to a nitrocellulose/nylon filter and immobilised. The relative positions of the DNA fragments in the gel are preserved during their transfer to the filter. After transfer, the DNA attached to the filter are hybridised to a labelled DNA probe and autoradiographed to pinpoint the location of any complementary bands.

Blotting was carried out using the procedure detailed in *Membrane Transfer And Detection Methods* (Amersham, U.K.). After electrophoresis and thorough destaining, unused areas of the gel were trimmed off with a razor blade. The DNA was denatured by soaking in several volumes of 1.5 M NaCl and 0.5 M NaOH (3 x 20 min) then in 0.25 M HCl (2 x 15 min) at r.t. with constant shaking. The gel was then neutralised by soaking in several volumes of 1 M Tris-HCl (pH 8.0) and 1.5 M NaCl for 1 hour again at r.t. with constant shaking.

For the transfer, a glass plate (bigger and wider than the gel) and four plastic lids were used as a support. A piece of overlapping Whatman 3MM paper was wrapped round the glass plate before being placed inside a container. The buffer reservoir was filled almost to the top of the support with 6x SSC buffer (**Appendix D**) so that the ends of the filter-paper wick were immersed in the buffer and then the air bubbles under the 3MM paper were smoothed out with a glass rod. The gel was inverted so that the underside was now on top and placed on the damp 3MM paper, ensuring that no air bubbles were trapped between the paper and the gel. A piece of 0.45 μm Hybond-N nylon filter (Amersham International plc., Amersham, U.K.), cut 2 mm larger than the gel in both dimensions, was carefully transferred on top of the gel so that the edge extended just over the line of slots. As before, trapped air bubbles were removed. Two pieces of 3MM paper, the same size as the filter, were soaked in 2x SSC and placed on top of the filter. A stack of paper

tissues just smaller than the 3MM paper, were placed on top. Finally, a glass plate was placed on top of the stack and weighed down with a 500 g weight. Nescofilm was used to surround the gel to prevent a short circuit of fluid from the paper tissues and the 3 MM paper under the gel.

The transfer was allowed to proceed for 16 h during which time the DNA fragments were eluted from the gel and deposited onto the filter. Transfer completed, the towels and 3MM above the gel were removed and the gel and filter turned over and placed gel side up on a dry sheet of 3MM paper. The slot positions were marked on the filter in ink before the gel was discarded. Any adhering agarose was removed by soaking in 2x SSC at r.t. for 5 min, then the filter was placed on a piece of 3MM paper to dry at r.t. The dry filter was wrapped in cling-film and placed, DNA-side down, on a U.V. transilluminator for 2 min (*Membrane Transfer And Detection Methods*, Amersham, U.K.). This induces the formation of covalent bonds thereby permanently bonding the DNA to the nylon. The filter was then wrapped in cling-film and stored in the dark at 4°C between sheets of 3MM paper.

2.9.6. Preparation of probes pKan2 and pSUP202

(a) Excision of probe insert from plasmids

Probes were obtained by digesting plasmids pKan2 and pSUP202 with the restriction enzymes HindIII (pKan2), BamHI and EcoRI (pSUP202). Electrophoresis and preparation of the probe for labelling was carried out using the modified method of Feinberg and Vogelstein (1983 and 1984) which involved the use a low-melting point agarose gel (1% in 1x TAE) (BRL, Life Technologies Inc., USA). Use of this type of agarose overcomes the need to phenol extract the probe from the gel.

After electrophoresis and staining, the desired bands were excised in the minimum amount of agarose from the gel and placed in an Eppendorf tube. Distilled water, 3 ml

per gram of agarose, was added and boiled for 7 min to dissolve the gel and denature the DNA. The probes were then labelled with ^{32}P using the random priming procedure.

(b) Random priming

Labelling of the probe was carried out using the method of Feinberg and Vogelstein (1983). The following solutions were added in order to an Eppendorf: Analar H_2O to make 50 µl, 10 µl reaction mix (**Appendix D**), 1 µl of 20 mg ml^{-1} nuclease-free BSA, 50 ng DNA solution, 30 µCi [$^{32}P\alpha$]-dCTP (specific activity 3000 Ci $mmol^{-1}$, Du Pont) and 1 µl (2-3 units) Klenow fragment. This was left to incubate at r.t. overnight.

(c) Purification of probe over Sephadex

Random priming completed, the DNA was separated from the unincorporated dCTP label using a Sephadex G50F (Pharmacia) gel filtration column on which the large DNA molecules i.e. the radiolabelled probe, are eluted earlier than the smaller unincorporated deoxynucleotides.

The sterilised Sephadex was poured into a 1 ml syringe, pre-plugged with siliconised glass wool, and packed down with the syringe plunger. Two column volumes of 1x TE buffer were run over the Sephadex to equilibrate the column. Then, using a moistened pipette tip, a tiny spot of Orange G (BDH Ltd., Poole, England), which co-migrates with unincorporated nucleotides, was added to the reaction vial, mixed and loaded onto the column. The column was eluted with 1x TE buffer and 3 drop aliquot fractions collected until the Orange G was eluted. Five microlitres from each fraction were counted using Cherenkov Counting (1 min per sample) to determine the activity. Cherenkov counting does not require the use of scintillant and has an efficiency of approximately 30%. The total counts in each fraction were calculated and those containing the highest levels of radioactivity which were eluted before Orange G were pooled.

2.9.7. Pre-hybridisation of filter

After Southern blotting, the Hybond-N filter was prehybridised using the method detailed by Mason and Williams (1987). The filter was placed in a heat-sealable polythene bag, sealed on three sides and examined for leaks. Then 26 ml (1 ml of liquid per 10 cm^2 of filter) prehybridisation fluid at 68°C was poured into the bag. Pre-hybridisation fluid was prepared from the following solutions: 4x SET buffer (**Appendix D**), 10x Denhardt's solution (**Appendix D**), 0.1% SDS, 0.1% sodium pyrophosphate and 50 µg ml^{-1} denatured salmon sperm DNA (**Appendix D**). The presence of 10x Denhardt's solution and denatured DNA in the pre-hybridisation and hybridisation fluids aids significantly in the reduction of background by saturating binding sites on the filter which would otherwise result in non-specific hybridisation (Mason and Williams, 1987).

The bubbles were removed from the bag to ensure that the filter was completely bathed in the fluid. This was achieved by displacing them out of one corner of the bag using a ruler. The bag was then double sealed, checked for leaks and placed in a gently shaking water bath for 12 h at 68°C. The filter was placed in the liquid DNA-side down to avoid any remaining bubbles and examined to ensure that it could move freely.

2.9.8. Hybridisation

Due to random priming, it was necessary to denature the probe again prior to hybridisation. One tenth original volume of 3 M NaOH was added and left at r.t. for 5 min, then 1/5 original volume of 1 M Tris-HCl (pH 7.0) was added to neutralise the solution after which 1/10 original volume of 3 M HCl was added. Hybridisation was carried out using the method of Mason and Williams (1987). After denaturation, a little of the pre-hybridisation fluid was removed, mixed with the labelled probe and poured into the bag. Having displaced the bubbles, the bag was carefully resealed and placed

inside a second bag. As before, the filter was checked to see that it could move freely. The bag was returned to the water bath (68°C) and left to shake gently for 16 h until the probe had re-annealed.

The bag was opened at one corner and the probe discarded. The filter was immersed completely in 300 ml of 4x SET buffer and 0.1% SDS. The filter was swirled for 1 min and the liquid discarded. Care was taken not to let the filter dry as this would lead to an increased background in the final autoradiograph. The filter was then washed in a shaking water bath according to the following regime which ensured that only specific hybrids were stable. Solutions were brought to temperature ahead of time using a microwave oven.

1. 3x for 20 min in 3x SET, 0.1% SDS, 0.1% Na pyrophosphate at 68°C
2. 2x for 20 min in 1x SET, 0.1% SDS, 0.1% Na pyrophosphate at 68°C
3. 1x for 20 min in 0.1x SET, 0.1% SDS, 0.1% Na pyrophosphate at 68°C
4. 1x for 20 min in 4x SET at r.t.

To avoid the probe becoming bound irreversibly, the filter, while still damp, was sealed in cling-film.

The filter was marked with ink containing ^{32}P waste to ensure proper orientation, wrapped in cling-film and autoradiographed. The X-ray film was pre-flashed using a flash-gun and placed between the filter and an intensification screen with the flashed side of the film next to the screen. The film was exposed in a cassette at -70°C for 24 h after which time the film was developed.

2.9.9. Removal of probe and re-use of Hybond-N filters

During the course of these experiments the same filter was used sequentially to hybridise to two different probes. The first probe was removed using the following non-damaging procedure (*Membrane Transfer And Detection Methods*, p.35. Amersham, U.K.).

The filter was immersed in a solution of 0.4 M NaOH and incubated at 45°C for 30 min in a gently shaking water bath. Then the blot was transferred to a solution of 0.1x SSC, 0.1% (w/v) SDS, 0.2 M Tris-HCl (pH 7.5) and incubated, again with agitation, for 30 min at 45°C. After which, the damp filter was wrapped in cling-film and autoradiographed to verify that the probe had been completely removed.

CHAPTER 3

RESULTS

3.1. Growth of *Rhizobium phaseoli* strain RW4 in liquid culture

Strain RW4 (Dr. A. W. Johnston, John Innes Institute, Norwich), an extracellular polysaccharide (EPS) minus mutant of *R. phaseoli*, was investigated to find out whether the increase in optical density of culture more accurately paralleled the increase in cell numbers than in 8002, an EPS$^+$ bacterium. EPS production by 8002 was thought to be responsible for the continuing increase in O.D. of cultures with time, even though estimation of cell numbers gave sigmoidal growth curves. If this was the case with RW4, it would allow rapid and accurate calculation of cell numbers prior to mutagenesis with Tn5.

On solid medium, strain RW4 colonies appeared dry in contrast to the slimy, moist colonies of strain 8002. However, in defined liquid medium (**Fig. 3.1.**), RW4, like strain 8002, showed a continued increase in optical density and failed to reach a plateau. Visually, the liquid culture resembled strain 8002. Consequently, it was decided to continue work on the Tn5 mutagenesis using the original strain 8002, on which most other work had been carried out.

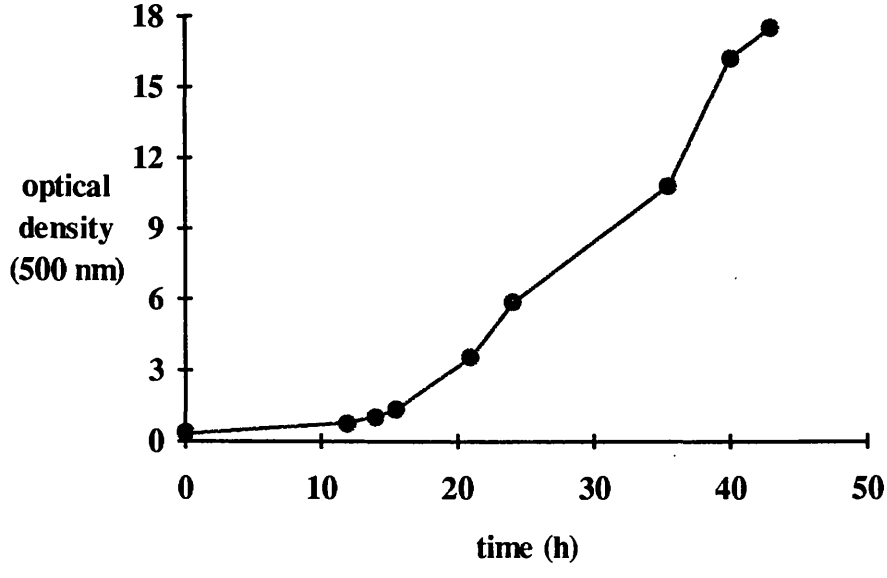


Fig. 3.1. Growth of *R. phaseoli* strain RW4 cultures, determined as the increase in optical density at 500 nm, in defined liquid medium shaken (120 rpm) at 27°C.

3.2. Analysis of indole standards by reversed-phase HPLC

The data presented in **Table 3.1.** show the retention times of a number of indole standards on four RP-HPLC regimes, isocratically at 50% **(a)** and 35% **(b)** and on a gradient of 25-70% methanol over 30 min **(c)** and 30-70% methanol over 30 min **(d)**. The separation of 14 of these indole standards on a gradient of 30-70% methanol over 30 min can be seen on **Fig. 3.2.**

Indole standard	Isocratic 50% (a)	Isocratic 35% (b)	Gradient 1 (c)	Gradient 2 (d)
IAAsp	3.9	6.4	7.6	8.2
IAAm	4.0	6.7	-	8.6
OxIAA	4.1	7.0	8.6	9.4
ILA	5.0	11.5	12	12.8
ICA	5.5	12.0	-	13.5
IAld	5.6	13.4	-	15
IAA	5.6	15.3	14.5	15.5
IEt	6.0	15.4	14.8	16
IAN	6.6	16.2	-	17.6
IAcetone	7.0	17.4	17.6	18.4
IPA	8.0	22.8	-	19
IM	8.7	24.4	19.4	20.8
I	9.3	-	-	21.4
IBA	10.5	25.7	23.6	26.2
Tryptophan	13.5	-	25.5	27.4
Tryptamine	12.0	-	-	29.1

Table 3.1. The RP-HPLC retention times (min) of 16 indole standards.

Isocratic and gradient elution RP-HPLC retention times of indole standards analysed on a 250 x 5 mm i.d. ODS Hypersil column with varying ratios of methanol in 1% acetic acid at a flow rate of 1 ml min^{-1}. Mobile phase conditions **a)** 50% methanol, **b)** 35% methanol, **c)** 30 min, 25-70 methanol gradient and **d)** 30 min, 30-70% methanol gradient. Detector: absorbance monitor at 280 nm.

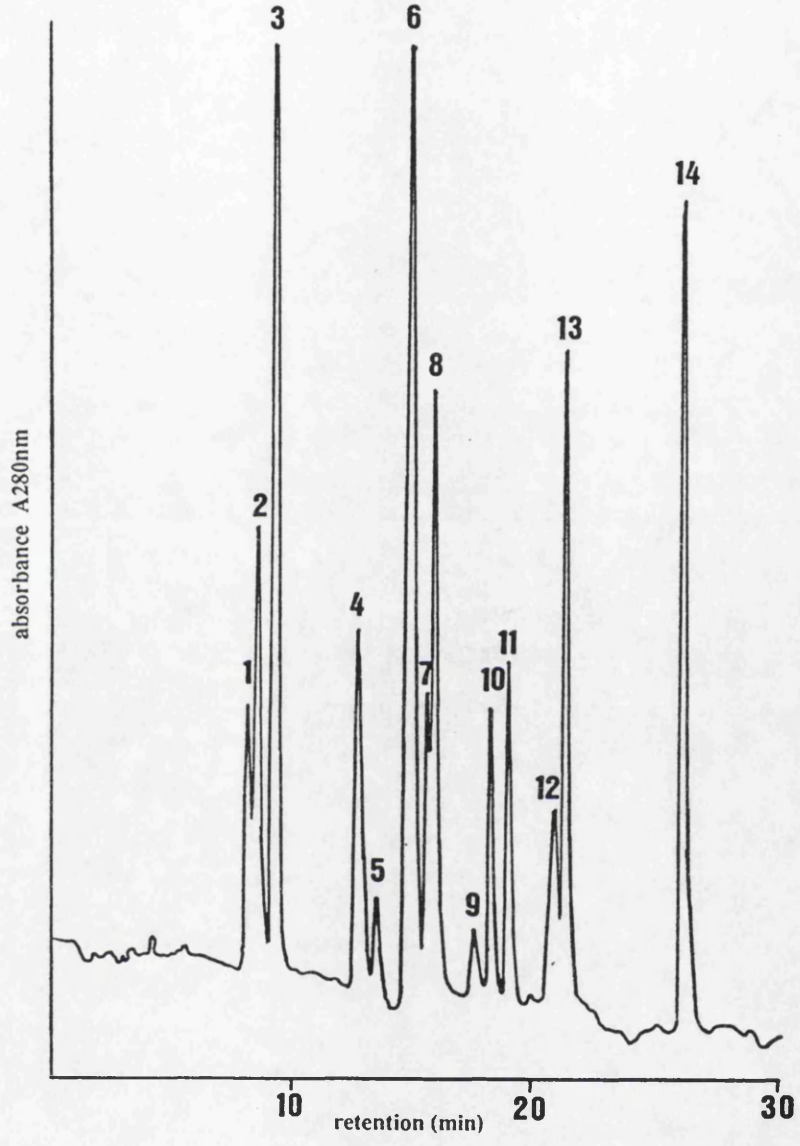


Fig. 3.2. Reversed-phase HPLC separation of 14 indole standards on a 30 min, 30-70% methanol (in 1% acetic acid) gradient at a flow rate of 1 ml min^{-1}. Column: ODS Hypersil 5µm, 250 x 5 mm i.d. Detector: absorbance monitor at 280 nm.

Indoles separated:

1. IAAsp
2. IAAm
3. OxIAA
4. ILA
5. ICA
6. IEt
7. IAld
8. IAA
9. IAN
10. indole acetone
11. IPA
12. IM
13. indole
14. IBA

It was found that IPyA, IAAld and tryptamine are unstable and break down readily. As a consequence of this, they have not been included in the analysis shown in **Fig. 3.2.** Unlike the other indoles, IAld does not fluoresce (Burnett and Audus, 1964) hence a U.V. absorbance monitor, rather than a fluorimeter, was used for the detection of the indoles in **Fig. 3.2.**

When the standards were run isocratically at 50% (**a**), the retention times of the indoles were similar, particularly those with the shortest retention times. IAAsp, IAAm and OxIAA were all eluted between 3.9 and 4.1 min and ILA, ICA, IAld and IAA between 5.0 and 5.6 min. Clearly, a better system of separation was required. Running the standards at 35% methanol (**b**) increased the retention times of all the indoles as expected. The separation was a little better than before, however it was difficult to distinguish between IAA (15.3 min) and IEt (15.4 min). In addition, tryptophan and tryptamine had very long retention times (>45 min). On gradients 1 and 2, all standards were eluted before the end of the gradient and they were reasonably well spread (particularly gradient 2) enabling each to be distinguished as a separate peak. Gradient 2 (30 min, 30-70% methanol) (**Fig. 3.2.**) was subsequently used for the majority of RP-HPLC analyses in this project.

3.3. Identification of indoles produced by *R. phaseoli* 8002 in tryptophan plus and minus medium

3.3.1. Analysis of the indoles produced by *R. phaseoli* 8002 *in vitro* by reversed and normal phase HPLC

Co-chromatography of the main HPLC peaks with authentic indole standards on both reversed (**Figs. 3.3.3. - 3.3.10.**) and normal phase (**Figs. 3.3.11. - 3.3.14.**) HPLC systems led to tentative identifications of the indoles produced by *R. phaseoli* in both tryptophan (+) (200 mg l^{-1}) and (-) medium (**2.2.3.**).

Non-biological conversion of tryptophan to IAA has been reported by a number of authors (Kaper and Veldstra, 1958; McDougall and Hillman, 1978; Epstein *et al.*, 1980; Badenoch-Jones *et al.*, 1982a) and highlights the problems of working with IAA which is readily oxidised in the presence of light, oxygen and peroxides (Ernstsen *et al.*, 1986). Indolic compounds however, were not detected in the acidic (**Fig. 3.3.1**) or neutral/basic (**Fig. 3.3.2.**) control extracts from either (+) or (-) tryptophan flasks (medium minus *Rhizobium*). It can be assumed therefore, that the extraction and partitioning techniques used did not cause significant breakdown of tryptophan to IAA, or any other indole, in the culture medium.

Analysis of the tryptophan (+) and (-) culture medium of *R. phaseoli* by RP-chromatography resulted in the detection of two major peaks in the acidic fraction and two in the neutral/basic fraction. From positive co-chromatography with authentic standards, the peaks in the acidic fraction were identified as indole lactic acid (ILA) (**Fig. 3.3.3.** and **3.3.4.**) and IAA (**Fig. 3.3.5.** and **3.3.6.**), and those from the neutral/basic fraction as indole ethanol (IEt) (**Fig. 3.3.7.** and **3.3.8.**) and indole methanol (IM) (**Fig. 3.3.9** and **3.3.10.**). The spectrum of indoles detected in both (+) and (-) tryptophan medium was therefore identical, although the quantities found in the tryptophan-supplemented medium were clearly larger (**Table 3.5.** and **3.6.**).

Further evidence of the identity of these compounds was obtained from co-chromatography of these peaks with authentic standards on a normal-phase chromatographic system (**2.5.1.**): ILA (**Fig. 3.3.11.**), IAA (**Fig. 3.3.12.**), IEt (**Fig. 3.3.13.**) and IM (**Fig. 3.3.14.**). It was found that although ILA fluoresces in reversed-phase solvents, in normal phase solvents it does not fluoresce and therefore a U.V. absorbance monitor was used to detect ILA (**2.5.2.**). These results are summarised in **Table 3.2.**

Fig. 3.3.1. Reversed-phase HPLC analysis of the acidic fractions obtained from (a) plus and (b) minus tryptophan control (minus *Rhizobium*) flasks.
Column: 250 x 5 mm i.d. ODS Hypersil (5 µm). Mobile phase: 30-70% methanol over 30 min in 1% acetic acid. Flow rate: 1 ml min^{-1}. Detector: fluorimeter, excitation 280 nm, emission 350 nm.

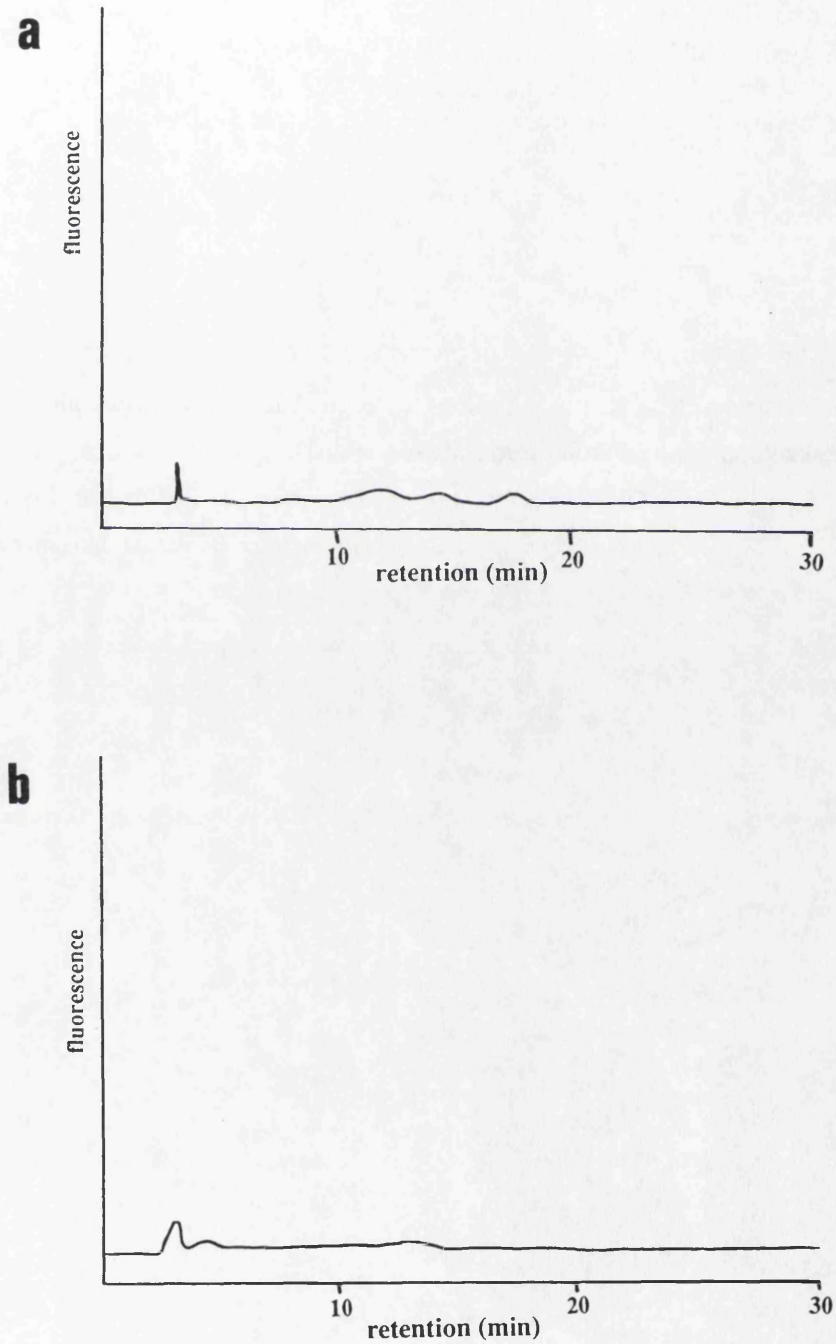

a
fluorescence
10
20
30
retention (min)
b
fluorescence
10
20
30
retention (min)

Fig. 3.3.2. Reversed-phase HPLC analysis of the neutral/basic fractions obtained from (a) plus and (b) minus tryptophan control (minus *Rhizobium*) flasks.

Column: 250 x 5 mm i.d. ODS Hypersil (5 µm). Mobile phase: 30-70% methanol over 30 min in 0.5% acetic acid (pH 6.5). Flow rate: 1 ml min^{-1}. Detector: fluorimeter, excitation 280 nm, emission 350 nm.

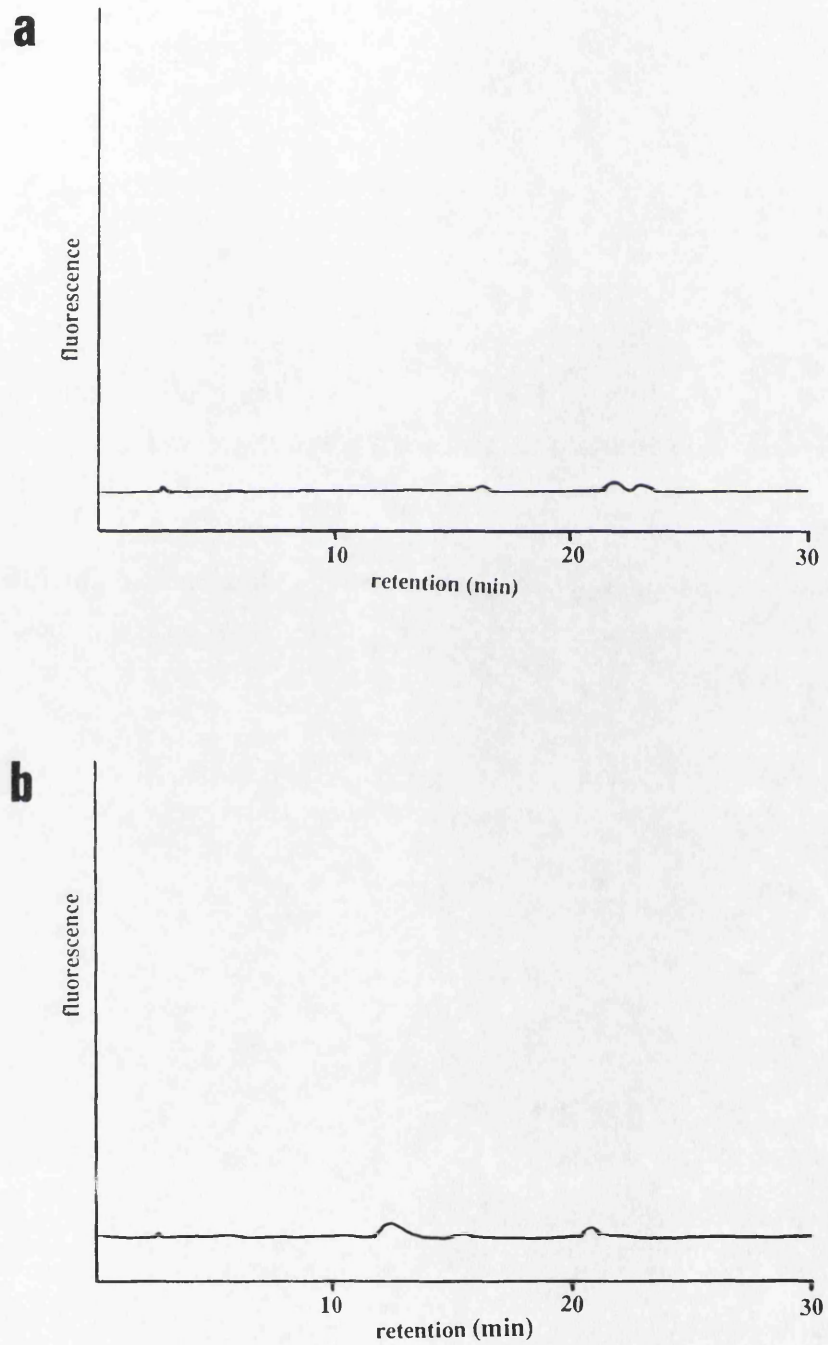

a
fluorescence
10
20
30
retention (min)
b
fluorescence
10
20
30
retention (min)

Fig. 3.3.3. Reversed-phase HPLC analysis of the acidic indoles produced by *R. phaseoli* 8002 in tryptophan (+) culture medium.

(a) putative ILA peak from *Rhizobium* culture medium, **(b)** authentic ILA standard and **(c)** co-chromatography of putative ILA peak with authentic ILA.

Column: 250 x 5 mm i.d. ODS Hypersil (5 μm). Mobile phase: 30-70% methanol over 30 min in 1% acetic acid. Flow rate: 1 ml min^{-1}. Detector: fluorimeter, excitation 280 nm, emission 350 nm.

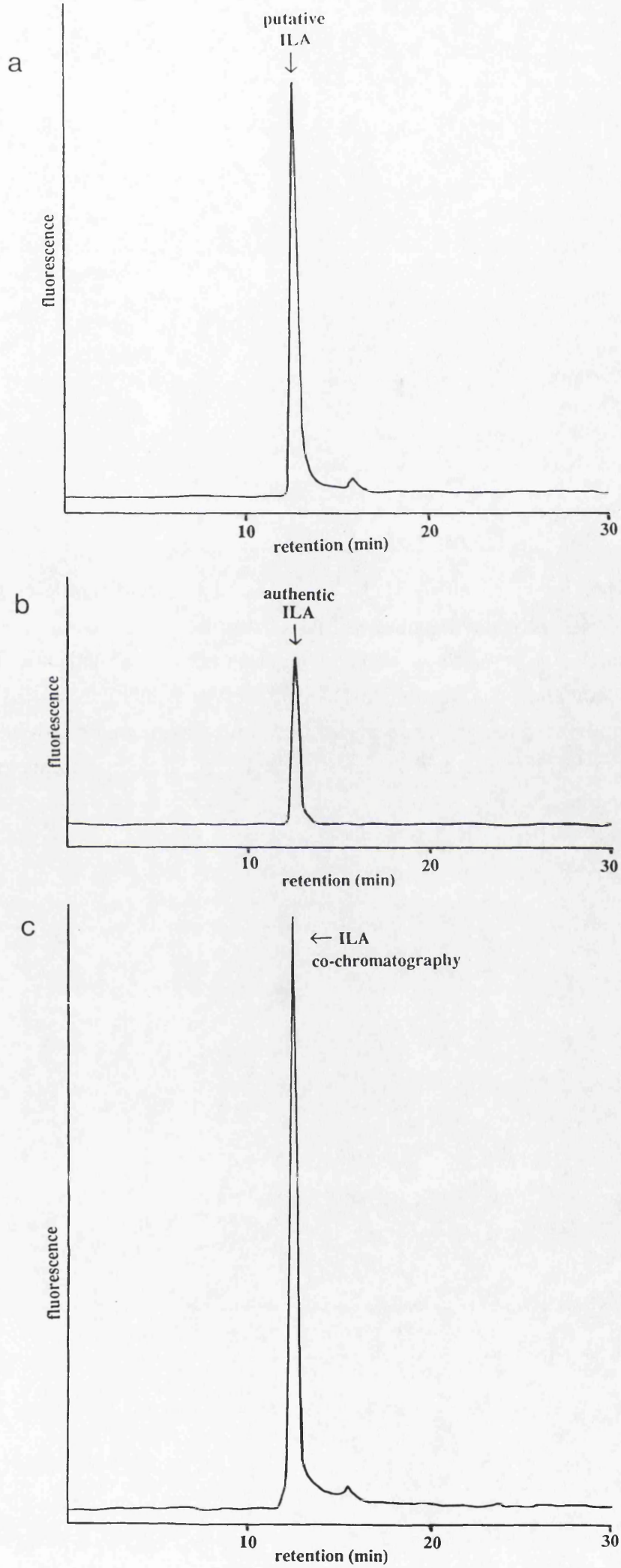

a
putative
ILA
fluorescence
10
20
30
retention (min)
b
authentic
ILA
fluorescence
10
20
30
retention (min)
c
← ILA
co-chromatography
fluorescence
10
20
30
retention (min)

Fig. 3.3.4. Reversed-phase HPLC analysis of the acidic indoles produced by *R. phaseoli* 8002 in (-) tryptophan culture medium.

(a) putative ILA peak from *Rhizobium* culture medium, **(b)** authentic ILA standard and **(c)** co-chromatography of putative ILA peak with authentic ILA.

Column: 250 x 5 mm i.d. ODS Hypersil (5 µm). Mobile phase: 30-70% methanol over 30 min in 1% acetic acid. Flow rate: 1 ml min^{-1}. Detector: fluorimeter, excitation 280 nm, emission 350 nm.

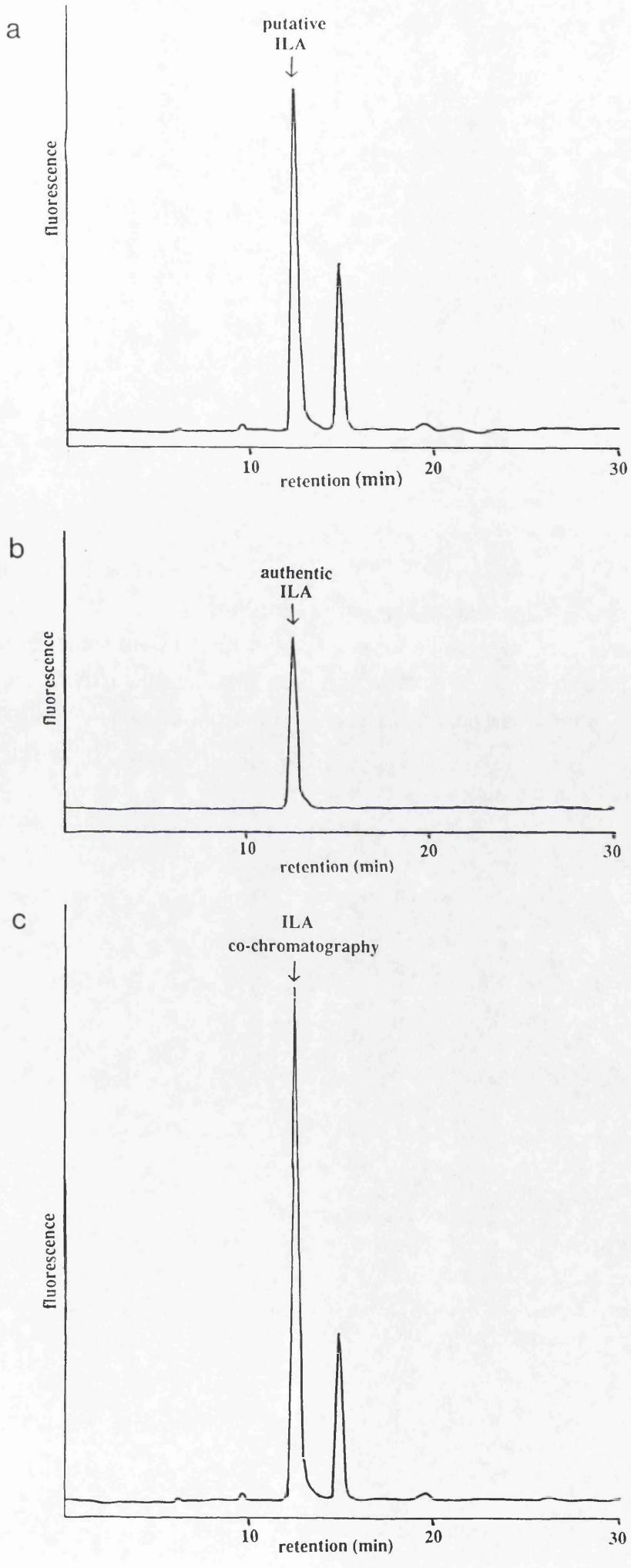

a
putative
ILA
fluorescence
10
20
30
retention (min)
b
authentic
ILA
fluorescence
10
20
30
retention (min)
c
ILA
co-chromatography
fluorescence
10
20
30
retention (min)

Fig. 3.3.5. Reversed-phase HPLC analysis of the acidic indoles produced by *R. phaseoli* 8002 in (+) tryptophan culture medium.

(a) putative IAA peak from *Rhizobium* culture medium, **(b)** authentic IAA standard and **(c)** co-chromatography of putative IAA peak with authentic IAA.

Column: 250 x 5 mm i.d. ODS Hypersil (5 µm). Mobile phase: 30-70% methanol over 30 min in 1% acetic acid. Flow rate: 1 ml min^{-1}. Detector: fluorimeter, excitation 280 nm, emission 350 nm.

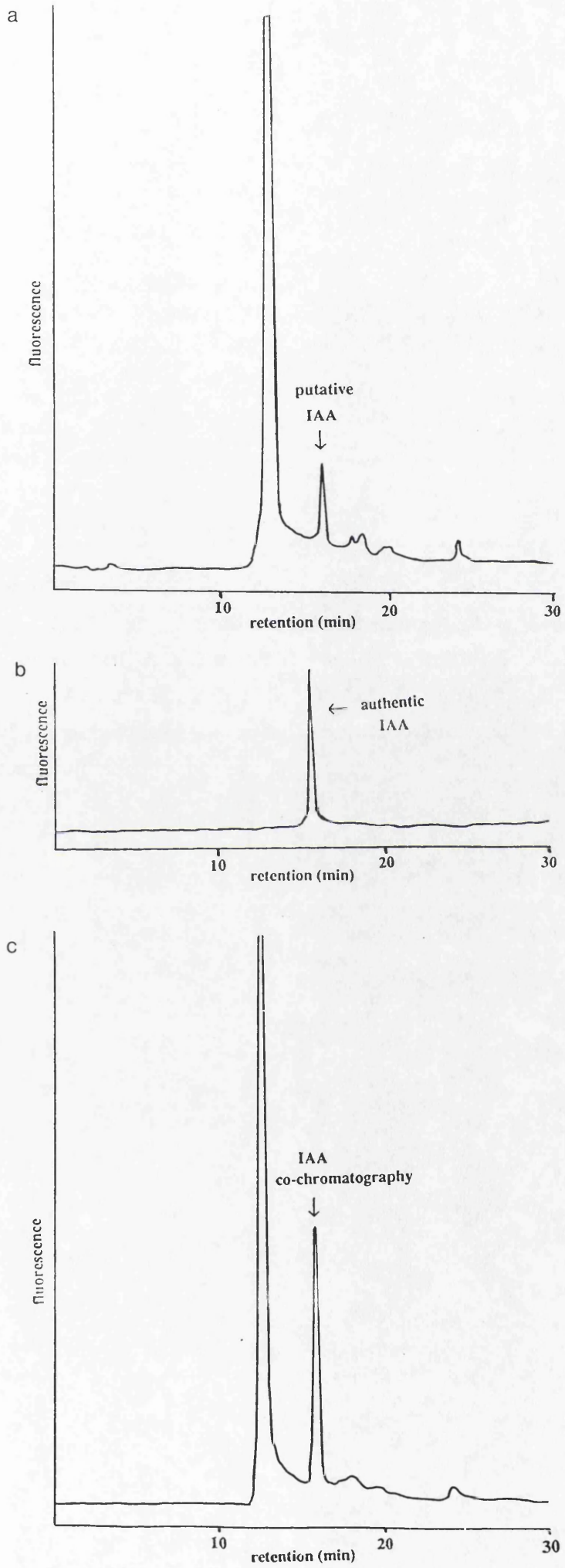

a
fluorescence
putative
IAA
10
20
30
retention (min)
b
fluorescence
authentic
IAA
10
20
30
retention (min)
c
fluorescence
IAA
co-chromatography
10
20
30
retention (min)

Fig. 3.3.6. Reversed-phase HPLC analysis of the acidic indoles produced by *R. phaseoli* 8002 in (-) tryptophan culture medium.

(a) putative IAA peak from *Rhizobium* culture medium, **(b)** authentic IAA standard and **(c)** co-chromatography of putative IAA peak with authentic IAA.

Column: 250 x 5 mm i.d. ODS Hypersil (5 μm). Mobile phase: 30-70% methanol over 30 min in 1% acetic acid. Flow rate: 1 ml min^{-1}. Detector: fluorimeter, excitation 280 nm, emission 350 nm.

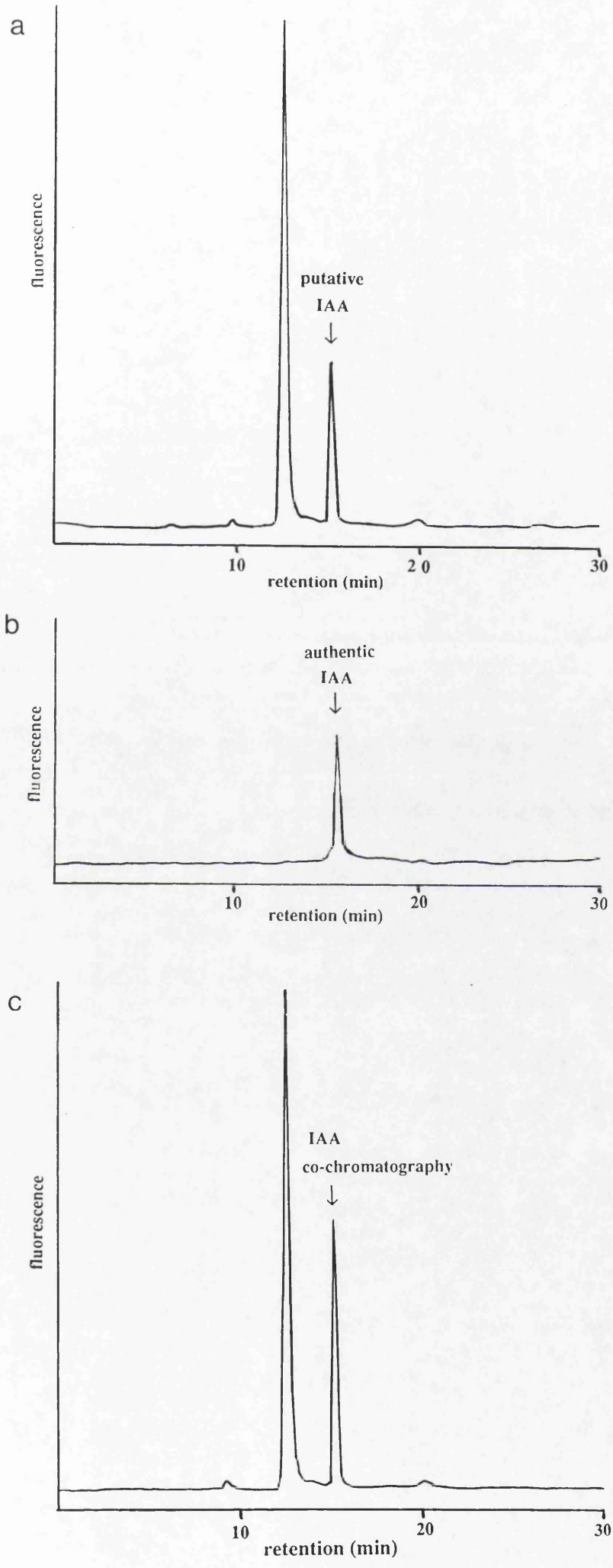

a
fluorescence
putative
IAA
10
20
30
retention (min)
b
fluorescence
authentic
IAA
10
20
30
retention (min)
c
fluorescence
IAA
co-chromatography
10
20
30
retention (min)

Fig. 3.3.7. Reversed-phase HPLC analysis of the neutral/basic indoles produced by *R. phaseoli* 8002 in (+) tryptophan culture medium.

(a) putative IEt peak from *Rhizobium* culture medium, **(b)** authentic IEt standard and **(c)** co-chromatography of putative IEt peak with authentic IEt.

Column: 250 x 5 mm i.d. ODS Hypersil (5 µm). Mobile phase: 30-70% methanol over 30 min in 0.5% acetic acid (pH 6.5). Flow rate: 1 ml min^{-1}. Detector: fluorimeter, excitation 280 nm, emission 350 nm.

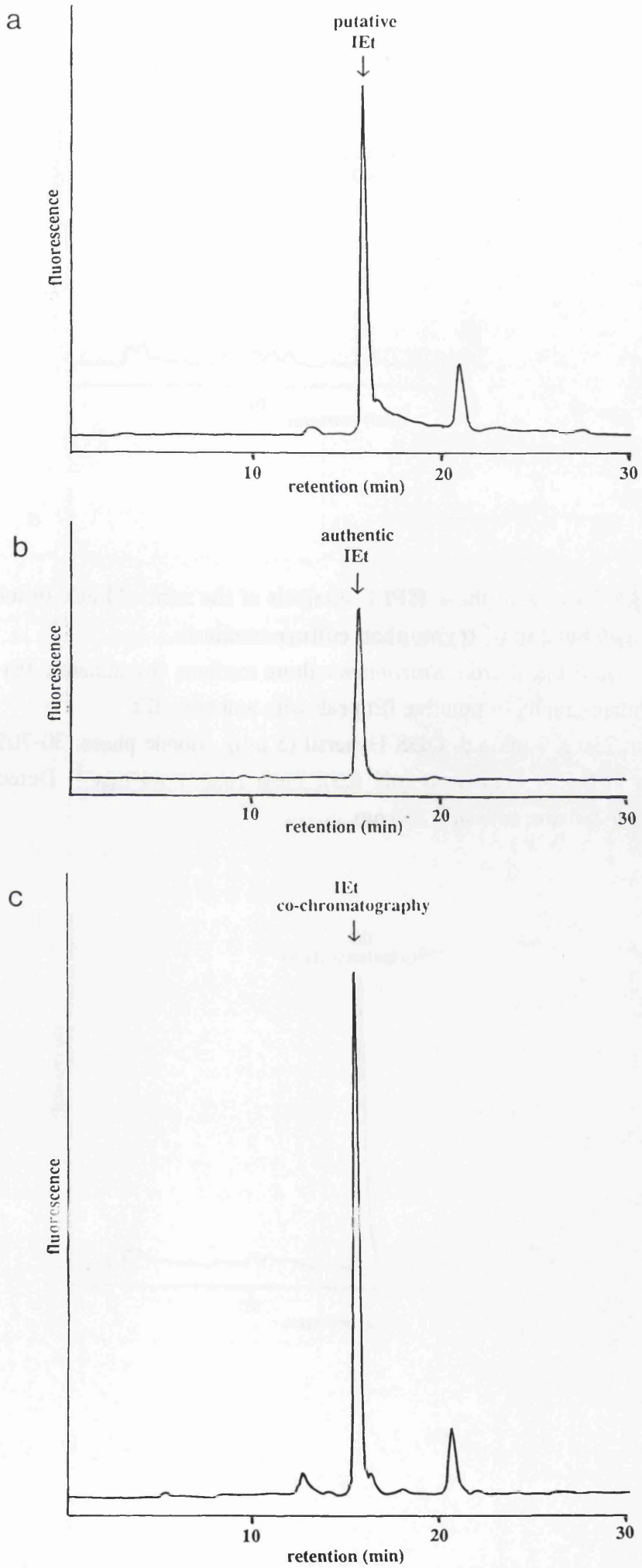

a
putative
IEt
fluorescence
10
20
30
retention (min)
b
authentic
IEt
fluorescence
10
20
30
retention (min)
c
IEt
co-chromatography
fluorescence
10
20
30
retention (min)

Fig. 3.3.8. Reversed-phase HPLC analysis of the neutral/basic indoles produced by *R. phaseoli* 8002 in (-) tryptophan culture medium.

(a) putative IEt peak from *Rhizobium* culture medium, **(b)** authentic IEt standard and **(c)** co-chromatography of putative IEt peak with authentic IEt.

Column: 250 x 5 mm i.d. ODS Hypersil (5 µm). Mobile phase: 30-70% methanol over 30 min in 0.5% acetic acid (pH 6.5). Flow rate: 1 ml min^{-1}. Detector: fluorimeter, excitation 280 nm, emission 350 nm.

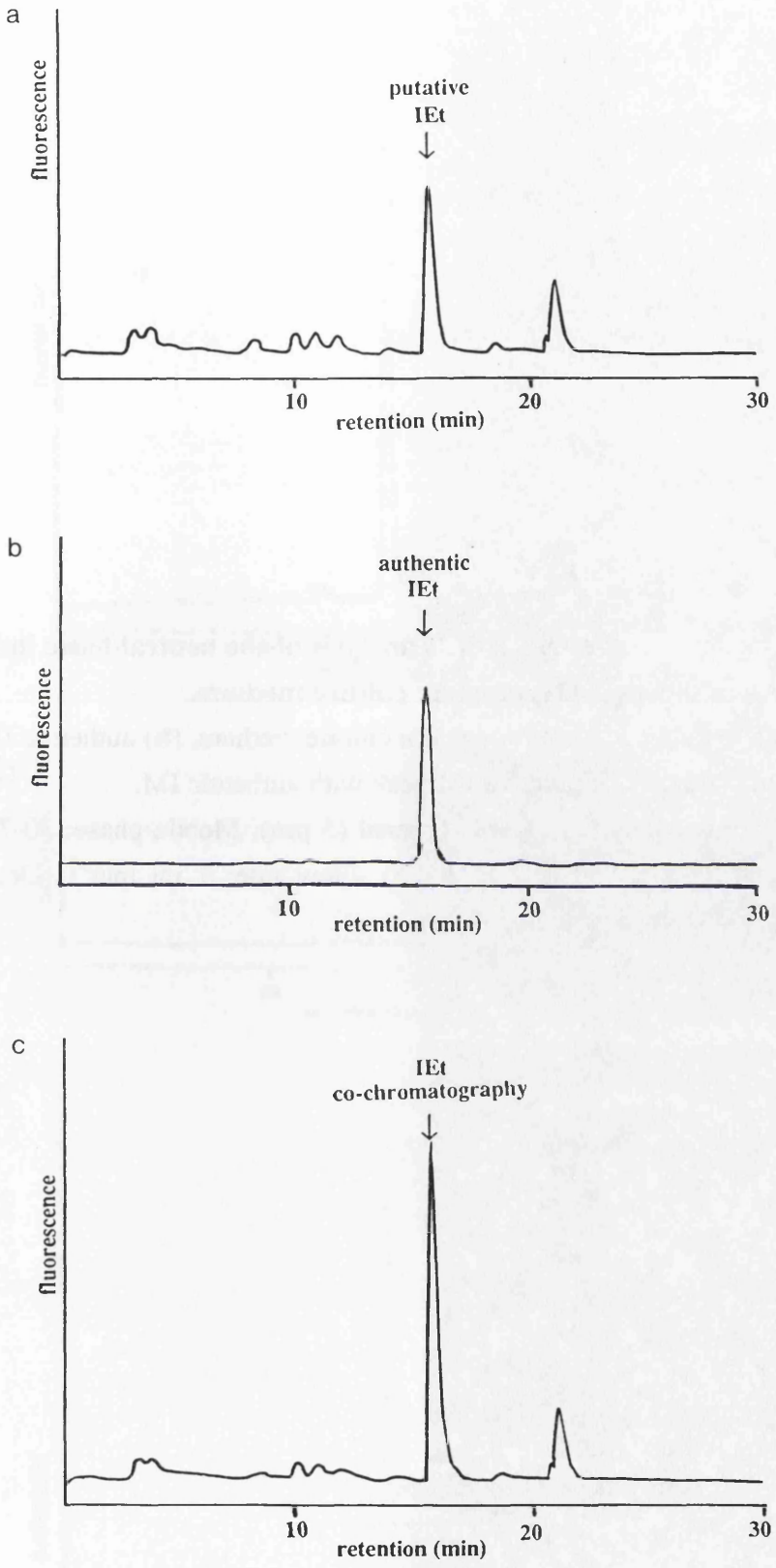

a
fluorescence
putative
IEt
10
20
30
retention (min)
b
fluorescence
authentic
IEt
10
20
30
retention (min)
c
fluorescence
IEt
co-chromatography
10
20
30
retention (min)

Fig. 3.3.9. Reversed-phase HPLC analysis of the neutral/basic indoles produced by *R. phaseoli* 8002 in (+) tryptophan culture medium.

(a) putative IM peak from *Rhizobium* culture medium, **(b)** authentic IM standard and **(c)** co-chromatography of putative IM peak with authentic IM.

Column: 250 x 5 mm i.d. ODS Hypersil (5 µm). Mobile phase: 30-70% methanol over 30 min in 0.5% acetic acid (pH 6.5). Flow rate: 1 ml min^{-1}. Detector: fluorimeter, excitation 280 nm, emission 350 nm.

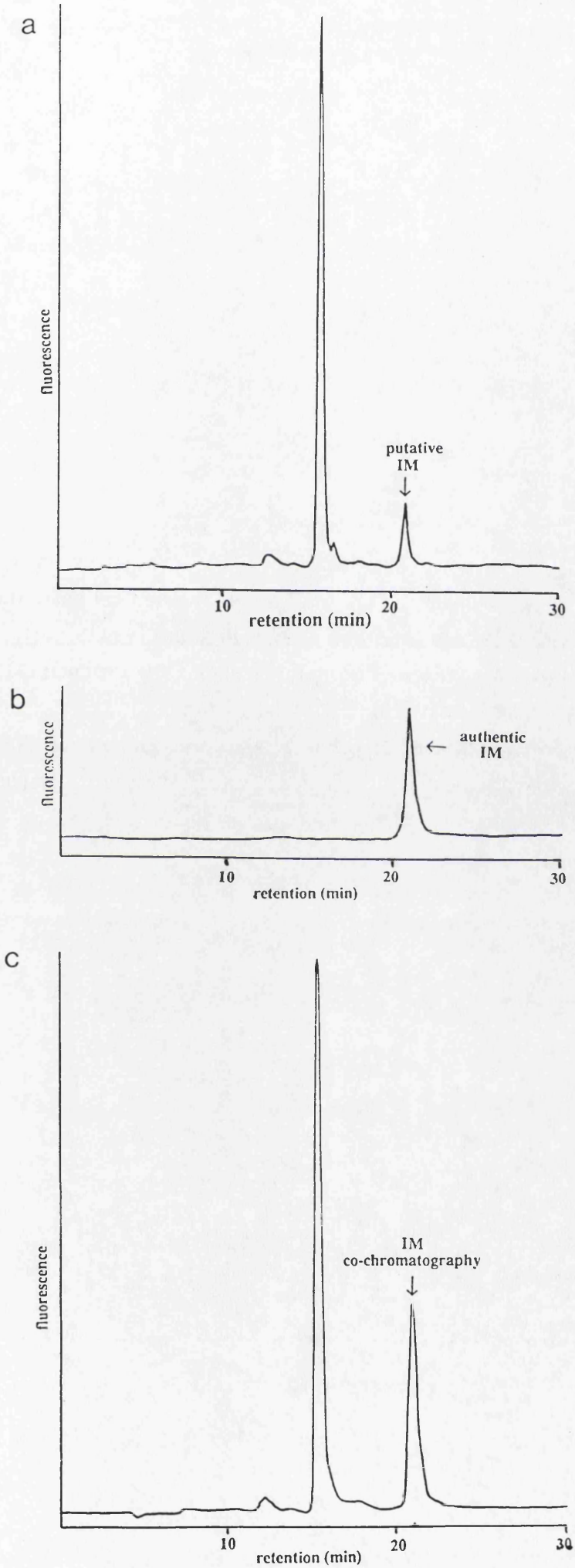

a
fluorescence
putative
IM
10
20
30
retention (min)
b
fluorescence
authentic
IM
10
20
30
retention (min)
c
fluorescence
IM
co-chromatography
10
20
30
retention (min)

Fig. 3.3.10. Reversed-phase HPLC analysis of the neutral/basic indoles produced by *R. phaseoli* 8002 in (-) tryptophan culture medium.

(a) putative IM peak from *Rhizobium* culture medium, **(b)** authentic IM standard and **(c)** co-chromatography of putative IM peak with authentic IM.

Column: 250 x 5 mm i.d. ODS Hypersil (5 μm). Mobile phase: 30-70% methanol over 30 min in 0.5% acetic acid (pH 6.5). Flow rate: 1 ml min^{-1}. Detector: fluorimeter, excitation 280 nm, emission 350 nm.

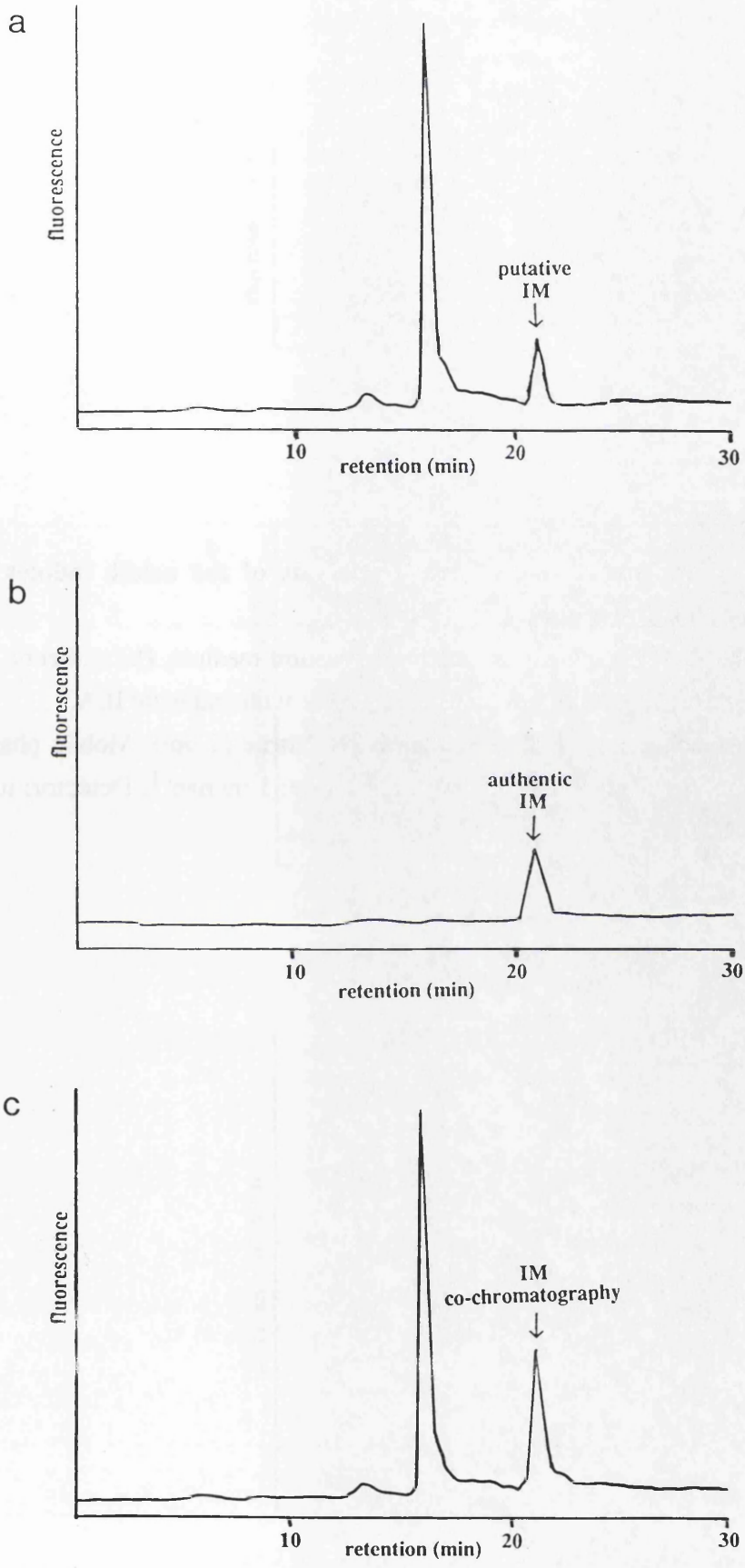
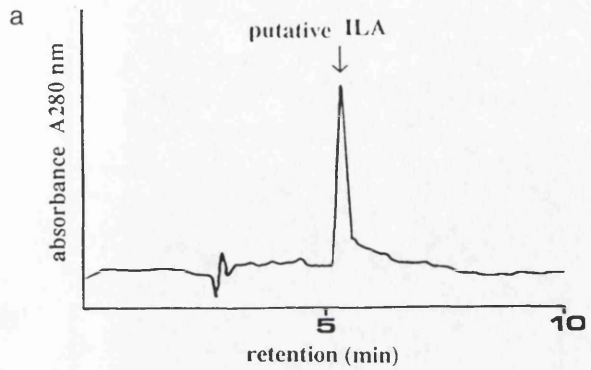
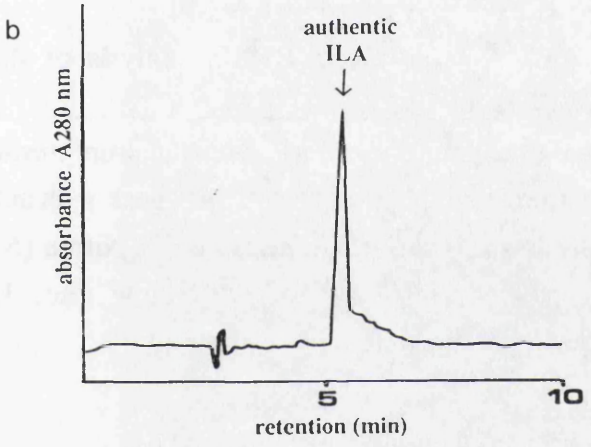

a
fluorescence
putative
IM
10
20
30
retention (min)
b
fluorescence
authentic
IM
10
20
30
retention (min)
c
fluorescence
IM
co-chromatography
10
20
30
retention (min)

Fig. 3.3.11. Normal-phase HPLC analysis of the acidic indoles produced by *R. phaseoli* 8002 in culture.

(a) putative ILA peak from *Rhizobium* culture medium, **(b)** authentic ILA standard and **(c)** co-chromatography of putative ILA peak with authentic ILA.

Column: 250 x 5 mm i.d. Spherisorb CN Nitrile (5 µm). Mobile phase: *n*-hexane/ethyl acetate/acetic acid (80:20:0.5, v/v). Flow rate: 1 ml min^{-1}. Detector: ultra-violet monitor set at 280 nm.


a
putative ILA
absorbance A280 nm
5
10
retention (min)


b
authentic
ILA
absorbance A280 nm
5
10
retention (min)

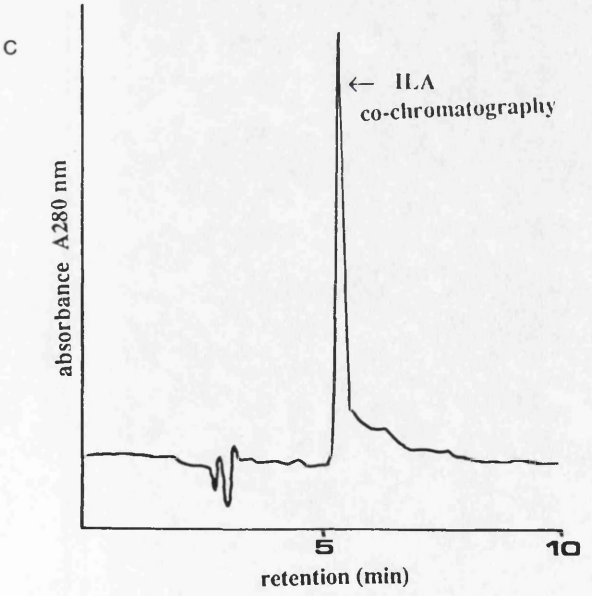

c
← ILA
co-chromatography
absorbance A280 nm
5
10
retention (min)

Fig. 3.3.12. Normal-phase HPLC analysis of the acidic indoles produced by *R. phaseoli* 8002 in culture.

(a) putative IAA peak from *Rhizobium* culture medium, **(b)** authentic IAA standard and **(c)** co-chromatography of putative IAA peak with authentic IAA.

Column: 250 x 5 mm i.d. Spherisorb CN Nitrile (5 µm). Mobile phase: *n*-hexane/ethyl acetate/acetic acid (60:40:0.5, v/v). Flow rate: 1 ml min^{-1}. Detector: fluorimeter, excitation 280 nm, emission 350 nm.

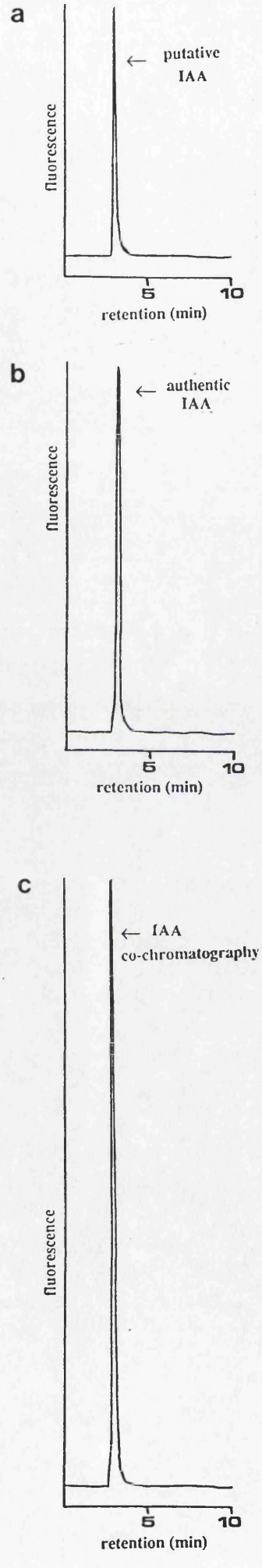

a
putative
IAA
fluorescence
5
10
retention (min)
b
authentic
IAA
fluorescence
5
10
retention (min)
c
IAA
co-chromatography
fluorescence
5
10
retention (min)

Fig. 3.3.13. Normal-phase HPLC analysis of the neutral/basic indoles produced by *R. phaseoli* 8002 in culture.

(a) putative IEt peak from *Rhizobium* culture medium, **(b)** authentic IEt standard and **(c)** co-chromatography of putative IEt peak with authentic IEt.

Column: 250 x 5 mm i.d. Spherisorb CN Nitrile (5 μm). Mobile phase: *n*-hexane/ethyl acetate/ethanol (65:35:1, v/v). Flow rate: 1 ml min^{-1}. Detector: fluorimeter, excitation 280 nm, emission 350 nm.

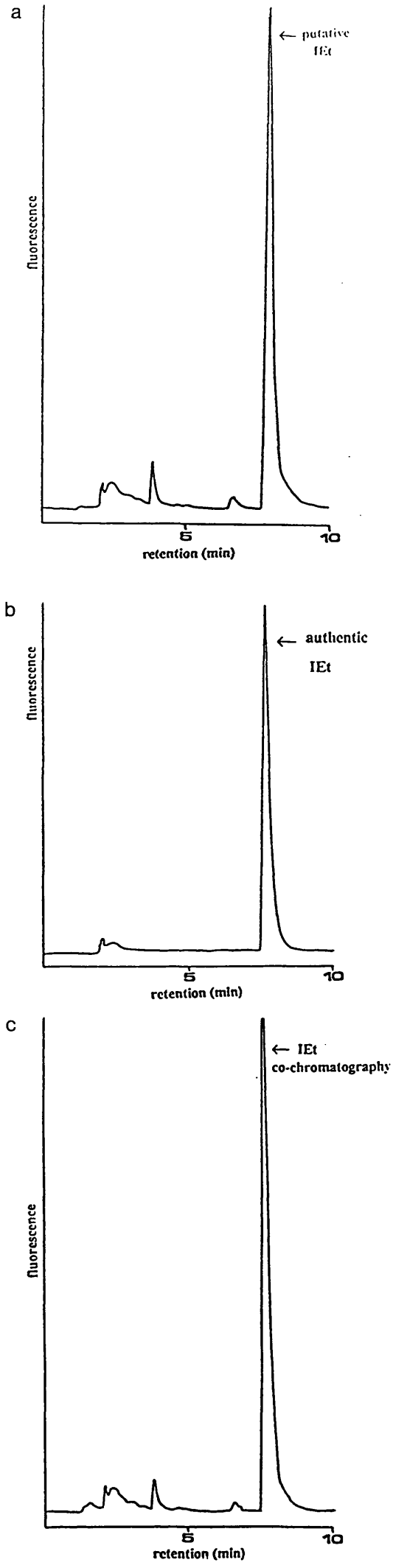
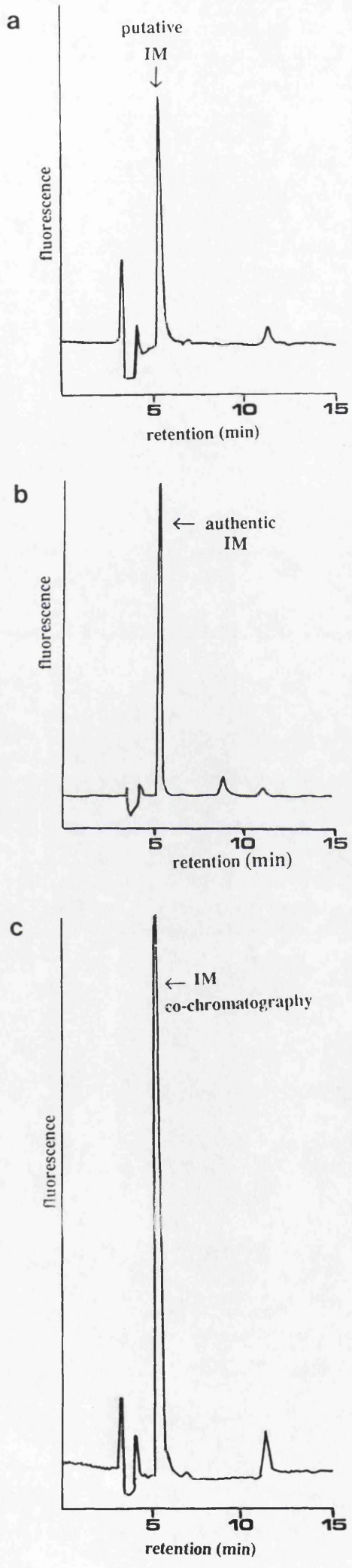

a
← putative
IEt
fluorescence
5
10
retention (min)
b
← authentic
IEt
fluorescence
5
10
retention (min)
c
← IEt
co-chromatography
fluorescence
5
10
retention (min)

Fig. 3.3.14. Normal-phase HPLC analysis of the neutral/basic indoles produced by *R. phaseoli* 8002 in culture.

(a) putative IM peak from *Rhizobium* culture medium, **(b)** authentic IM standard and **(c)** co-chromatography of putative IM peak with authentic IM.

Column: 250 x 5 mm i.d. Spherisorb CN Nitrile (5 µm). Mobile phase: *n*-hexane/ethyl acetate/ethanol (60:40:1, v/v). Flow rate: 1 ml min^{-1}. Detector: fluorimeter, excitation 280 nm, emission 350 nm.


a
putative
IM
fluorescence
5
10
15
retention (min)
b
← authentic
IM
fluorescence
5
10
15
retention (min)
c
← IM
co-chromatography
fluorescence
5
10
15
retention (min)

indoles detected	Tryptophan (+)	(-)
acidic	ILA, IAA	ILA, IAA
neutral/basic	IEt, IM	IEt, IM

Table 3.2. Summary of the indolic compounds identified in the (+) and (-) tryptophan culture medium of *R. phaseoli* 8002.

3.3.2. Analysis of the indoles produced by *R. phaseoli* 8002 *in vitro* by mass spectrometry

The putative indole peaks from reversed phase HPLC (**3.3.1.**) were purified by normal phase HPLC and analysed by mass spectrometry. The electron impact mass spectra total ion current traces of the purified extracts and the standards are shown in **Figs. 3.4.1. - 3.4.5. Table 3.3.** lists the % abundance relative to the base ion of the molecular ion, base peak and the other diagnostic ions of authentic indole standards (IAA, IEt, ILA and IM) and of the sample peaks. The ion fragmentation pattern, m/z 130 (quinolinium ion), 131, 103 (styryl ion) and 77 (phenyl ion), is characteristic for most 3-substituted indoles, specifically those with the general formula indole-3-CH_2-R (M^{c}Dougall and Hillman, 1978; Sandberg *et al.*, 1987).

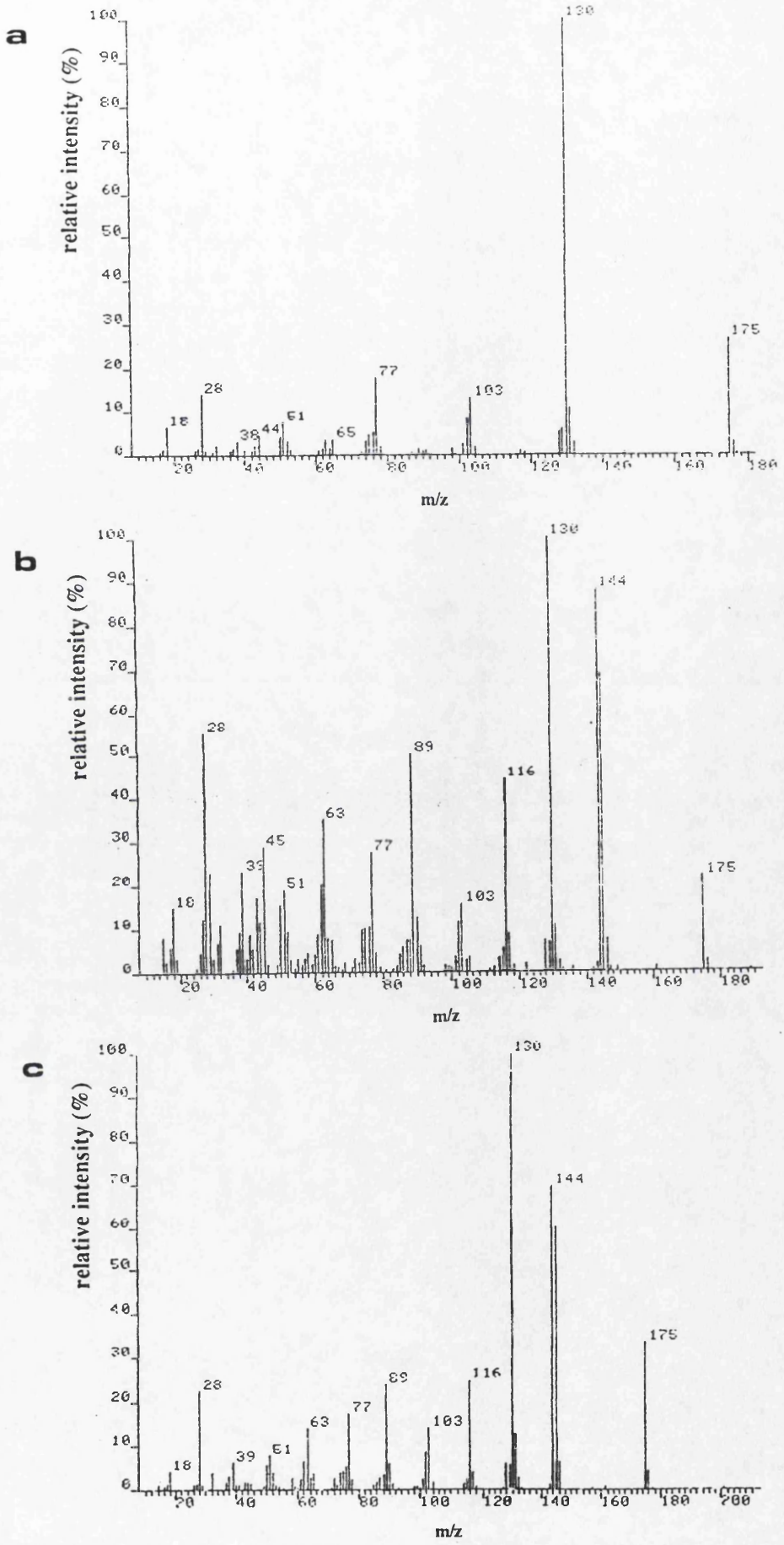


Fig. 3.4.1. Positive ion 70eV electron impact mass spectra of **(a)** authentic IAA, **(b)** putative IAA extracted from the culture medium of *R. phaseoli* 8002 and **(c)** authentic IAA/IAld mix.

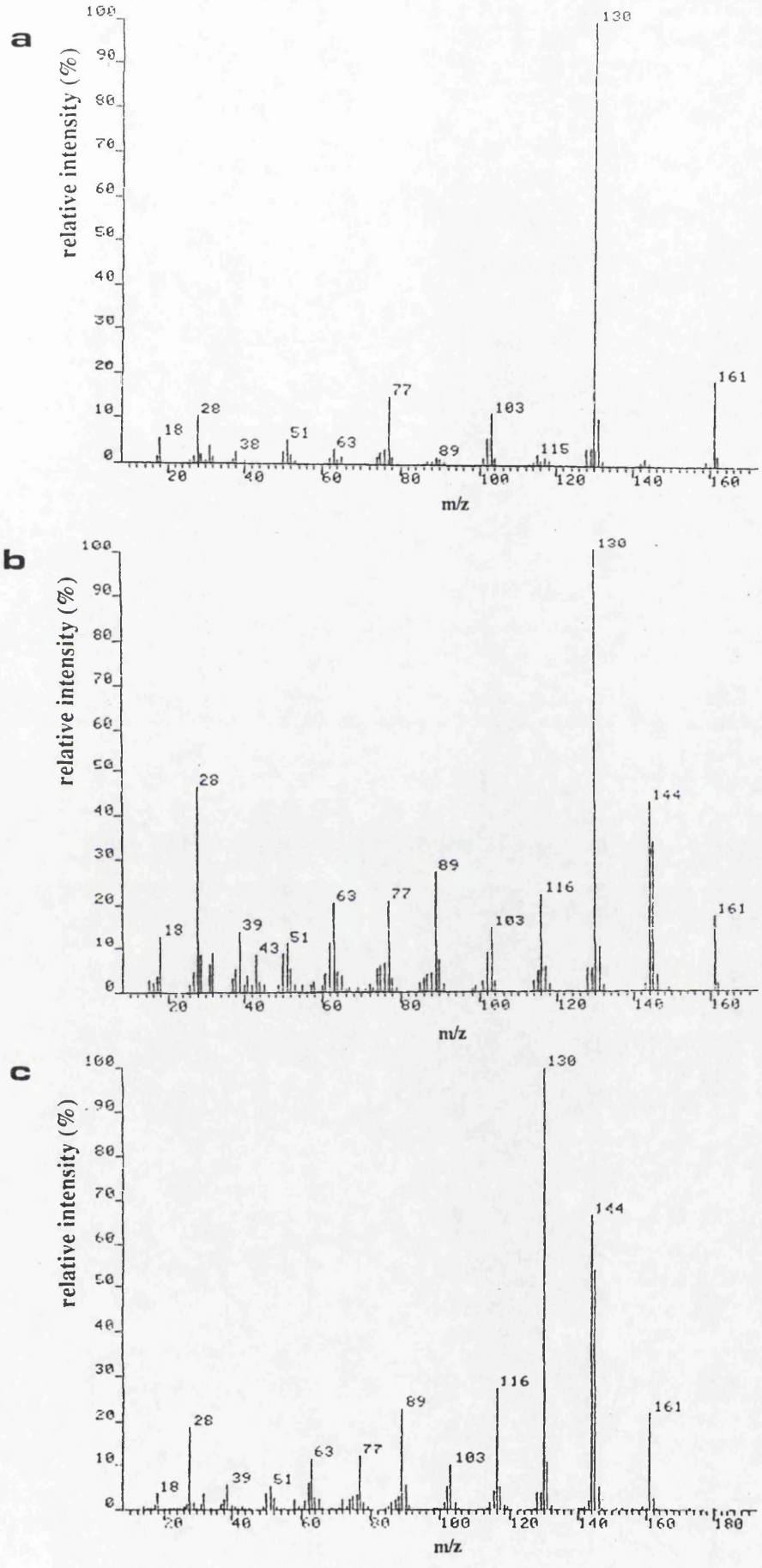


Fig. 3.4.2. Positive ion 70eV electron impact mass spectra of **(a)** authentic IEt, **(b)** putative IEt extracted from the culture medium of *R. phaseoli* 8002 and **(c)** authentic IEt/IAld mix.

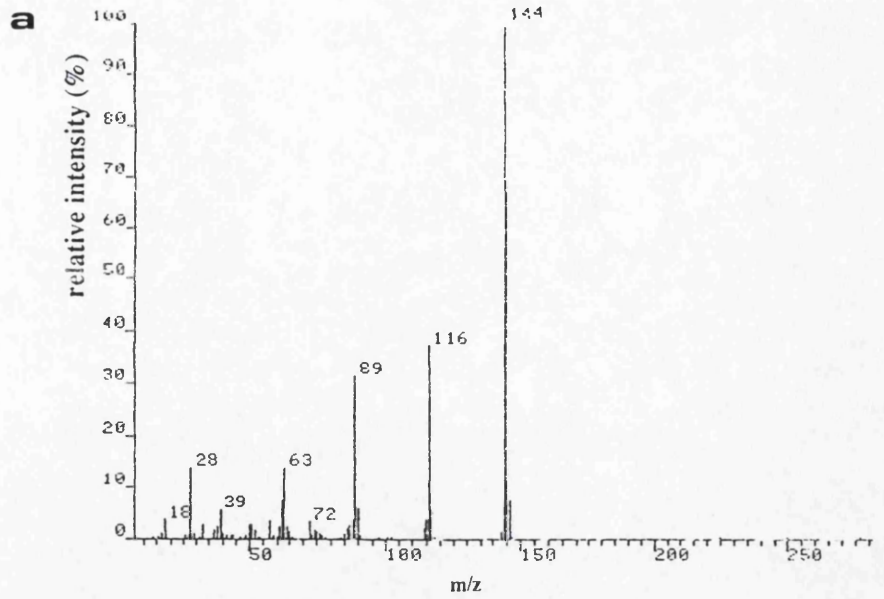


Fig. 3.4.3. Positive ion 70eV electron impact mass spectra of **(a)** authentic IAld.

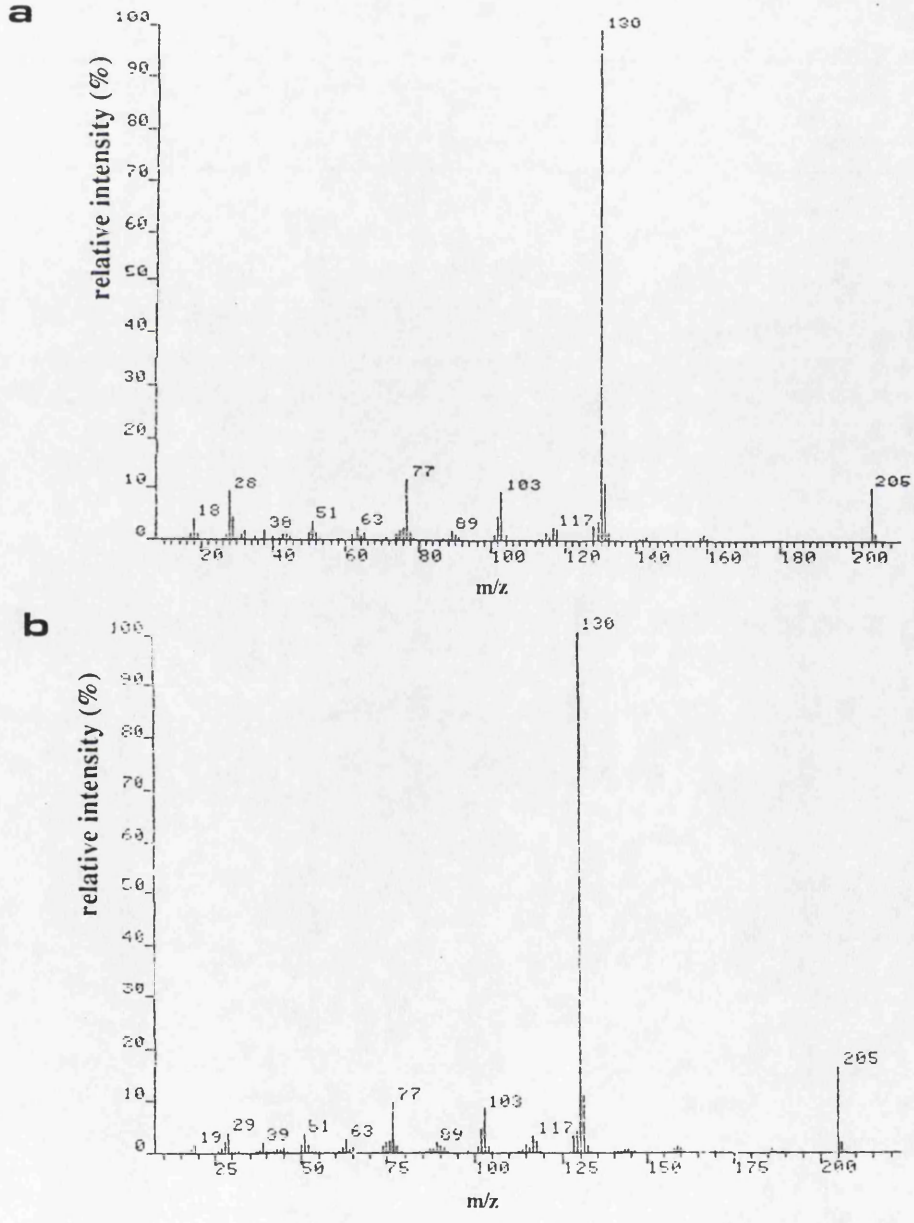


Fig. 3.4.4. Positive ion 70eV electron impact mass spectra of **(a)** authentic ILA and **(b)** putative ILA extracted from the culture medium of *R. phaseoli* 8002.

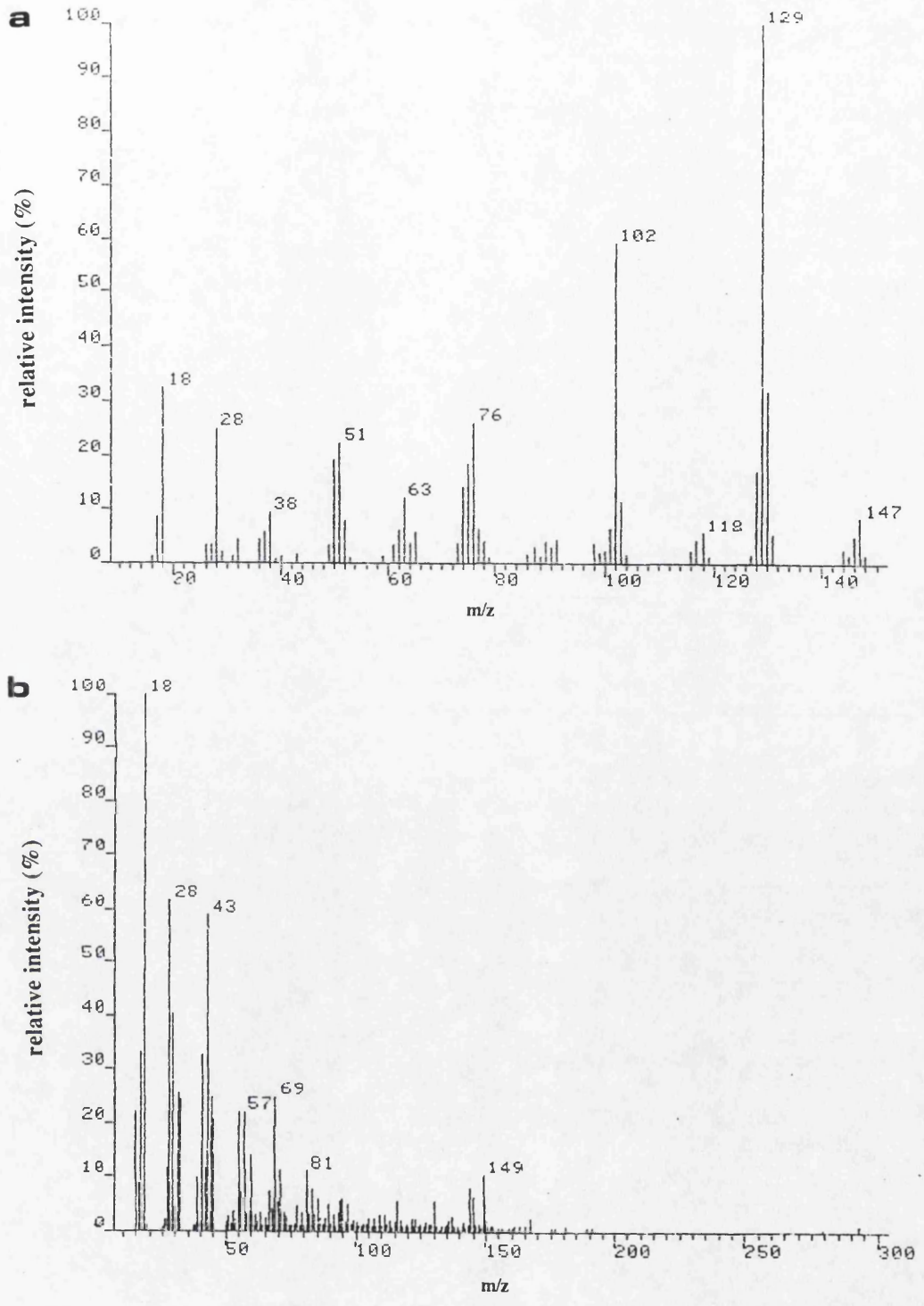


Fig. 3.4.5. Positive ion 70eV electron impact mass spectra of **(a)** authentic IM and **(b)** putative IM extracted from the culture medium of *R. phaseoli* 8002.

	diagnostic ions	% abundance relative to base ion (a) standard	(b) sample
IAA	175 (m^+)	26.5	21.8
	130 (base ion)	100.0	100.0
	131	10.7	10.7
	103	13.3	15.9
	77	17.9	27.6
IEt	161 (m^+)	18.9	17.2
	130 (base ion)	100.0	100.0
	131	10.5	10.3
	103	11.5	14.3
	77	14.9	20.6
ILA	205 (m^+)	10.5	16.4
	130 (base ion)	100.0	100.0
	131	10.9	11.1
	103	9.6	8.4
	77	12.1	9.7
IM	147 (m^+)	8.4	1.6
	130 (base ion)	31.9	5.8
	131	5.1	1.3
	103	11.5	0.0
	77	6.1	4.8

Table 3.3. The abundance of diagnostic ions relative to the base ion in the positive 70 eV mass impact spectra of **(a)** authentic IAA, IEt, ILA and IM **(b)** putative IAA, IEt, ILA and IM from purified extracts of *R. phaseoli* strain 8002 culture medium.

	% abundance relative to base ion				
diagnostic ions	(a) IAld standard	(b) IAld/IAA standard	(c) HPLC IAA peak	(d) IAld/IEt standard	(e) HPLC IEt peak
146	7.3	6.2	7.5	5.2	3.3
145	69.8	60.2	68.8	54.3	33.7
144	100.0	69.5	88.1	66.3	43.1
116	37.3	25.1	44.1	27.6	22.0
89	31.6	23.9	50.1	23.1	26.9

Table 3.4. Percentage abundance relative to base ion of diagnostic ions m/z 146, 145, 144, 116 and 89 of **(a)** authentic IAld standard (**Fig. 3.4.3a.**) **(b)** mixture of authentic IAld and IAA (**Fig. 3.4.1c.**) **(c)** putative IAA peak from HPLC analysis (**Fig. 3.4.1b.**) **(d)** mixture of authentic IAld and IEt (**Fig. 3.4.2c.**) **(e)** putative IEt peak from HPLC analysis (**Fig. 3.4.2b.**).

Co-chromatography of major HPLC peaks with standards (**Figs. 3.3.3. - 3.3.14.**) and the mass spectra of the peaks (**Fig. 3.4.1. - 3.4.5.**) suggest the following identities:

(i) The peak which co-chromatographed with IAA, gave a mass spectrum (**Fig. 3.4.1b.**) containing diagnostic ions characteristic of the standard (**Fig. 3.4.1a.**). The molecular ion (m/z 175), base peak (m/z 130) and other diagnostic ions (m/z 131, 103 and 77) were present at % abundances of the base ion similar to the standard (**Table 3.3.**). However, additional ions were detected with m/z values of 145, 144, 116, and 89, implying that the IAA peak was impure. Indolic compounds of the type indole-3-CO-R have an intense indole-3-CO^+ ion (m/z 144) which by loss of carbon monoxide gives the indolyl ion (m/z 116) (Sandberg *et al.*, 1987). This suggests that the major contaminant was probably another indole. The spectrum of ions produced by the unknown compound was similar to IAld (mol. wt.=145, m/z 144, 146, 116 and 89) (**Fig. 3.4.3a.** and **Table 3.4.**). **Fig. 3.4.1c.** shows the spectrum of ions produced when authentic IAA is contaminated by IAld (20% by volume). This spectrum closely resembles that of the putative IAA peak (**Fig. 3.4.1b.**) suggesting that this peak contains a mixture of IAA and IAld.

(ii) Mass spectra of the putative indole ethanol peak (**Fig. 3.4.2b.**) contained diagnostic ions with m/z 161, 130, 131, 103, 77 similar to authentic IEt (**Fig. 3.4.2a.**). In addition ions with m/z values of 145, 144, 116 and 89 were present and again, it is probable that this compound is indole aldehyde. **Fig. 3.4.2c.** shows the spectrum of an IEt and IAld mixture which is similar to the putative IEt peak (**Fig. 3.4.2b.** and **Table 3.4.**).

(iii) Putative indole lactic acid (**Fig. 3.4.4b.**) gave a mass spectrum very similar to the standard (**Fig. 3.4.4a.**). The characteristic base ion (m/z 130) and other diagnostic ions (m/z 205, 130, 131, 103, 77) were present at abundances similar to that of the standard (**Table 3.3.**).

(iv) Despite positive co-chromatography of this peak with authentic indole methanol (IM) on both reversed (**Figs. 3.3.9.** and **3.3.10.**) and normal phase HPLC systems (**Fig. 3.3.14.**), the putative IM (**Fig. 3.4.5b.**) gave a spectrum of ions which had limited similarity to that of the standard (**Fig. 3.4.5a.**) (**Table 3.3.**). This suggests that IM present in the sample is low in quantity and impure. Unfortunately, time did not allow for repetition of this analysis.

3.4. Quantification of indoles in the culture medium of *R. phaseoli* 8002 grown in tryptophan plus and minus medium

The data presented in **Table 3.5.** illustrates the accumulation of IAA, ILA, IEt and IM in tryptophan (+) (200 mg l^{-1}) and (-) culture medium of *Rhizobium*. The addition of tryptophan increased the accumulation of these indolic compounds, particularly IAA, ILA and IEt. This work was carried out before the discovery of IAld (**Fig. 3.4.**) in the culture medium and thus this indole has not been quantified.

Table 3.6. shows the multiplication factor per litre and per 10^{11} cells of the tryptophan minus medium. The amount of IAA in the supplemented flasks per litre culture medium and per 10^{11} rhizobial cells was 87 and 86 times that of the mean level which accumulated in the tryptophan (-) medium. The increase in IEt in the tryptophan supplemented medium was of a similar order to IAA, being 68 (per litre) and 69 (per 10^{11} cells) times that of the unsupplemented medium. There was even greater accumulation of ILA in the tryptophan supplemented medium with levels 237 and 240 times (per litre and per 10^{11} cells) greater than in tryptophan (-) medium. In contrast to the large effects of tryptophan supplementation on IAA, ILA and IEt accumulation, IM levels increased by a relatively small amount to 1.75 times (per litre and 10^{11} cells) the value found in unsupplemented medium.

tryptophan (+/-)	IAA		ILA		IEt		IM	
	µg per litre	µg per 10^{11} cells	µg per litre	µg per 10^{11} cells	µg per litre	µg per 10^{11} cells	µg per litre	µg per 10^{11} cells
- tryp. 1	21.5	2.9	82.1	11.2	9.4	1.3	7.4	1.0
- tryp. 2	20.3	2.8	58.5	8.0	7.6	1.0	9.8	1.3
+ tryp. 1	1537	235	17372	2652	591	90	11.8	1.8
+ tryp. 2	2093	258	15996	1970	563	69	17.9	2.2

Table 3.5. Levels of IAA, ILA, IEt and IM in the culture filtrate of *Rhizobium phaseoli* 8002 growing in plus and minus tryptophan medium. The amount (mean µg per litre and per 10^{11} cells) of IAA, ILA, IEt and IM extracted from the culture medium of *R. phaseoli* 8002 wild-type growing in tryptophan-supplemented (200 mg l^{-1}) and in non-supplemented medium in an orbital shaker (120 rpm) at 27°C. Variation between the values for duplicate analyses of aliquots from the same flask was less than 6 % for IAA, less than 11% for ILA, less than 9% for IEt and less than 15% for IM.

	Multiplication factor of tryp. (-) medium.	
	per litre	per 10^{11} cells
IAA	87	86
IEt	68	69
ILA	237	240
IM	1.75	1.75

Table 3.6. The multiplication factor per litre and per 10^{11} cells for IAA, IEt, ILA and IM of the tryptophan (-) medium.

The percentage content of individual indoles, expressed as a percentage of the total per litre and per 10^{11} cells is shown on **Table 3.7.** While tryptophan supplementation resulted in an increase in the relative amount of ILA accumulated in the medium (from 65 to 87% of the total percentage of indoles), the percentage of IAA, IEt and IM decreased, especially IM which fell substantially from 8% to less than 0.1% of the total.

Tryptophan (+/-)	IAA	ILA	IEt	IM
-	19%	65%	8%	8%
+	10%	87%	3%	<0.1%

Table 3.7. The content of individual indoles (IAA, ILA, IEt, and IM) expressed as a percentage of the total per litre and per 10^{11} cells.

3.5. The effect of gibberellic acid (GA_3) on IAA production by *R. phaseoli* 8002 grown in minus tryptophan liquid medium

The results from duplicate experiments are shown in **Table 3.8a.** and **3.8b.** In both experiments, the amounts of IAA produced in the control flasks were broadly similar,

(a)

Treatment +/- GA_3	IAA µg l^{-1}	IAA µg per 10^{11} cells
$-GA_3$	50.1	22.2
$-GA_3$	46.2	20.4
$-GA_3$	40.0	17.7
mean ± S.E. (n=3)	45.4 ± 2.9	20.1 ± 1.3
10^{-6} M GA_3	209.6	59.9
10^{-6} M GA_3	66.8	29.0
10^{-6} M GA_3	56.1	24.4
mean ± S.E. (n=3)	110.8 ± 49.5	37.8 ± 11.1
10^{-5} M GA_3	59.5	25.9
10^{-5} M GA_3	42.3	18.4
10^{-5} M GA_3	69.7	30.3
mean ± S.E. (n=3)	57.2 ± 8.0	24.9 ± 3.5

(b)

Treatment +/- GA_3	IAA µg l^{-1}	IAA µg per 10^{11} cells
$-GA_3$	16.7	7.6
$-GA_3$	21.3	9.7
$-GA_3$	61.6	28.0
mean ± S.E. (n=3)	33.2 ± 14.3	15.1 ± 6.5
10^{-6} M GA_3	21.8	7.5
10^{-6} M GA_3	6.8	2.3
10^{-6}M GA_3	37.2	12.8
mean ± S.E. (n=3)	21.9 ± 8.8	7.5 ± 3.0
10^{-5} M GA_3	17.0	8.5
10^{-5} M GA_3	23.9	11.9
10^{-5} M GA_3	18.5	9.2
mean ± S.E. (n=3)	19.8 ± 2.1	9.9 ± 1.0

Table 3.8a. and 3.8b. IAA accumulation in gibberellic acid supplemented culture medium of *R. phaseoli* 8002.

The mean amount of IAA (µg l^{-1} and per 10^{11} cells) extracted from the culture medium of *R. phaseoli* 8002. Cells were grown in gibberellic acid supplemented, tryptophan-free medium (10^{-5} and 10^{-6} M GA_3) in an orbital shaker (120 rpm) at 27°C until harvested. Three separate flasks were grown for each treatment. Values given are the mean of two duplicate analyses of sub-samples for each flask. Variation between analyses of duplicate sub-samples from the same flask in experiment **(a)** was between 6% and 14% and in experiment **(b)** between 12% and 24%.

with the mean production between the two experiments of 39.3 μg l^{-1} culture medium and 17.6 μg 10^{-11} live bacterial cells. The difference in control IAA production between experiments was not significant at $p \leq 0.05$. However, the effect on IAA production of supplying gibberellin (GA) was highly variable.

In experiment **(a)** (**Table 3.8a.**), an apparent increase in IAA production following treatment with 10^{-5} M GA, and particularly 10^{-6} M GA, was not statistically different from the control ($p \leq 0.05$). In experiment **(b)** (**Table 3.8b.**), IAA production apparently decreased following GA treatment but again mean values for production per litre and per 10^{11} cells were not significantly different from control values at $p \leq 0.05$.

3.6. Metabolism of [^{3}H]-tryptophan and [^{14}C]-IAA by cultures of *R. phaseoli* 8002

3.6.1. Metabolism of [^{3}H]-tryptophan

The products of the metabolism of [^{3}H]-tryptophan by cultures of *R. phaseoli* 8002 were investigated by RP-HPLC. The partitioning of the [^{3}H] label, after extraction into acidic, neutral/basic and aqueous fractions, is shown in **Table 3.9.**

flask	total % recovery	% of radioactivity recovered: acidic fraction	neutral/basic fraction	aqueous fraction
control	70	1.5	0.5	98
flask 1	65	18	6	76
flask 2	71	20.5	7.5	72

Table 3.9. Partitioning of [^{3}H]-labelled metabolites extracted from the culture medium of *R. phaseoli* 8002. The percentage of [^{3}H]-label which partitioned into the ethyl acetate acidic, neutral/basic and aqueous fractions after feeding *R. phaseoli* 8002 for 3 h with [^{3}H]-tryptophan. Cells were grown in an orbital shaker (120 rpm) in defined minimal medium plus 5 mg l^{-1} tryptophan at 27°C.

Total label recovery for all flasks was approximately 2/3 that of the radioactivity added initially. The majority (approximately 3/4) of the label recovered in the experimental flasks had partitioned into the aqueous fraction where it was associated with unmetabolised tryptophan. The percentage of radioactivity which partitioned into the acidic ethyl acetate fraction was greater than that which partitioned into the neutral/basic ethyl acetate fraction. In the control flask, 98% of the label recovered partitioned into the aqueous fraction, with a small percentage of label partitioning into the acidic and neutral/basic fraction (1.5 and 0.5% respectively), however, no radioactive peaks were found in the minus *Rhizobium* control acidic and neutral/basic extracts indicating firstly, that the extraction procedure was efficient and secondly, that the level of tryptophan decomposition and non-enzymatic conversion to, for example, IAA, was insignificant **(Fig. 3.5.1.)**.

Analysis of the acidic ethyl acetate fractions by RP-HPLC **(Fig. 3.5.2.)** revealed two major radioactive peaks with retention times similar to those of IAA and ILA. **Fig. 3.5.3.** shows the retention time of a radiolabelled IAA standard. The presence of these compounds was shown additionally by fluorescence monitoring. Analysis of the neutral/basic ethyl acetate fraction by RP-HPLC revealed three major radioactive peaks with retention times similar to IEt, IAld and IM **(Fig. 3.5.4.)**. When the fluorescence of the sample was monitored however, only two peaks were detected, one corresponding to IM and the other to IEt. This was to be expected as IAld cannot be detected by fluorescence **(2.5.2.)**. Both of these fluorescent peaks co-chromatographed successfully with authentic IEt and IM standards.

These results suggest that radiolabelled metabolites produced when cultures of *R. phaseoli* are incubated with [^{3}H]-tryptophan, summarised in **Table 3.10.**, appear to be identical to the indolic compounds which accumulate in tryptophan (+) and (-) culture medium **(3.3.)**.

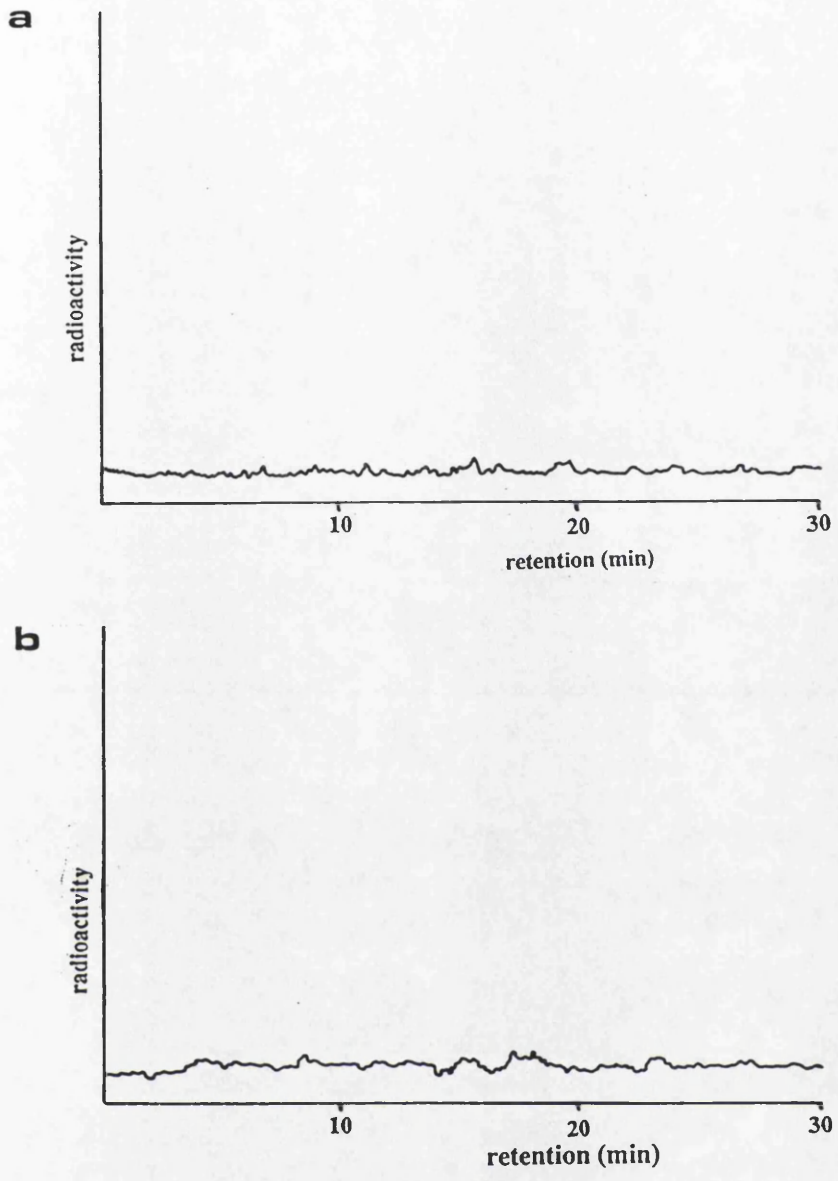


Fig. 3.5.1. Reversed-phase HPLC analysis of the (a) acidic fraction and (b) neutral/basic fraction obtained from the control (minus *Rhizobium*) flask after a 3 h incubation with [^{3}H]-tryptophan (120 rpm, 27°C).

Column: 250 x 5 mm i.d., ODS Hypersil (5 µm). Mobile phase: 30-70% methanol over 30 min in 1% acetic acid. Flow rate: 1 ml min^{-1}. Detector: Radioactivity monitor operating in the homogeneous mode (500 µl flow cell).

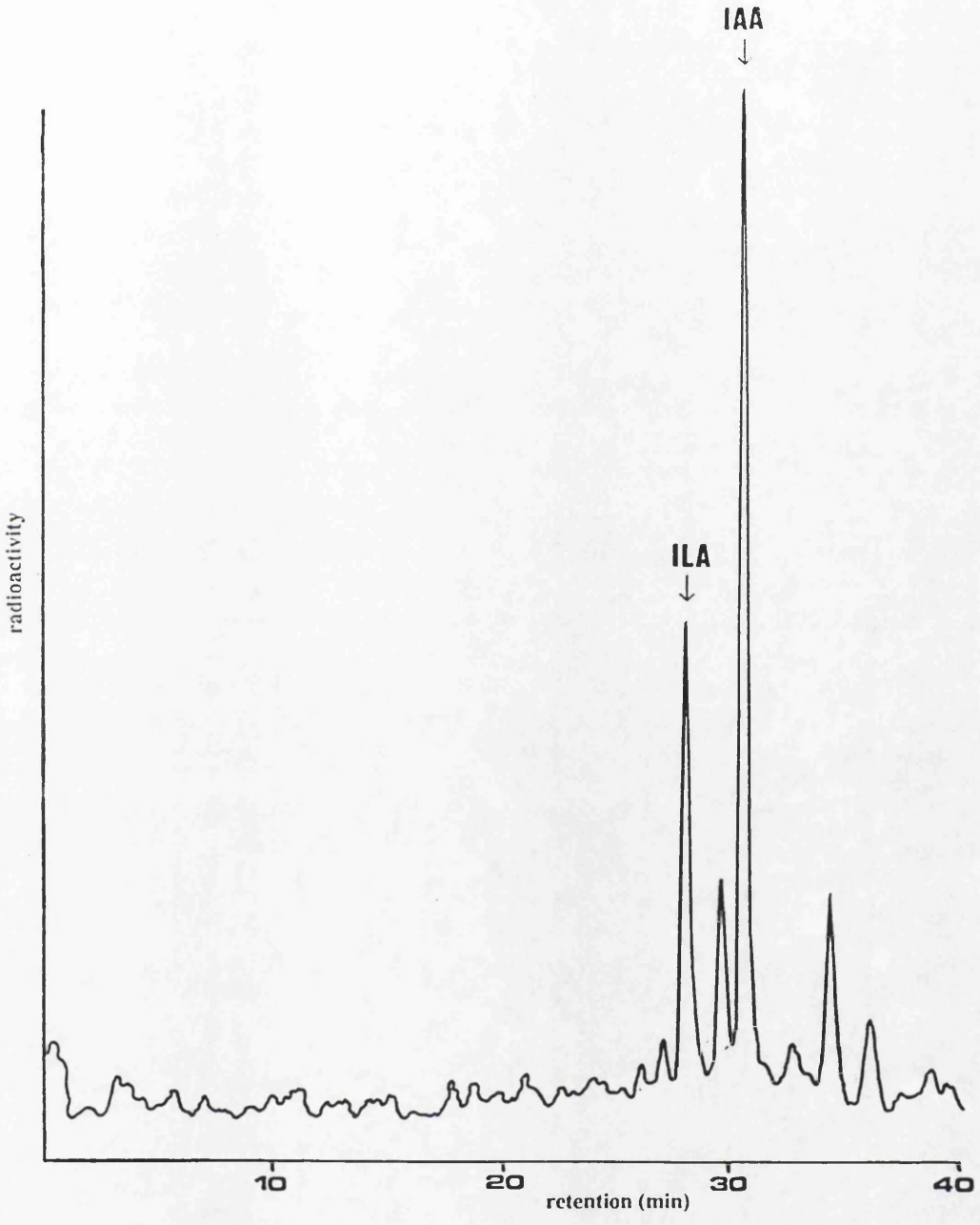


Fig. 3.5.2. Reversed-phase HPLC analysis of an aliquot (70 Kdpm) of the acidic ethyl acetate fraction obtained from the culture medium of *Rhizobium phaseoli* after a 3 h incubation with [^{3}H]-tryptophan (120 rpm, 27°C).

Column: 250 x 5 mm i.d., ODS Hypersil (5 µm). Mobile phase: methanol in 1% acetic acid, 0-5 min 10%, 5-30 min 10-60%. Flow rate: 1 ml min^{-1}. Detector: Radioactivity monitor operating in the homogeneous mode (500 µl flow cell).

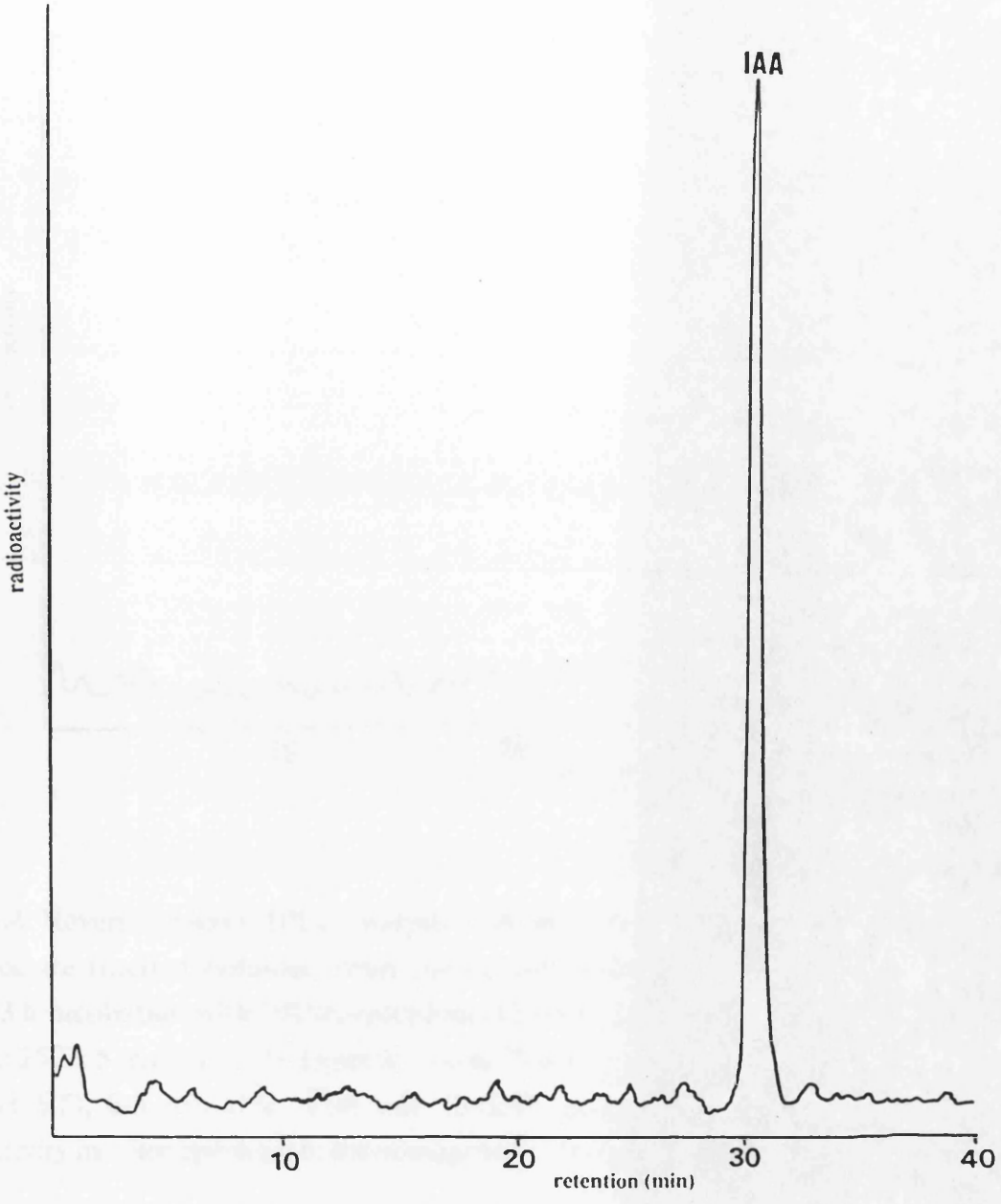


Fig. 3.5.3. Retention time of [^{14}C]-IAA standard (50 Kdpm) on reversed-phase HPLC.

Column: 250 x 5 mm i.d., ODS Hypersil (5 µm). Mobile phase: methanol in 1% acetic acid, 0-5 min 10%, 5-30 min 10-60%. Flow rate: 1 ml min^{-1}. Detector: Radioactivity monitor operating in the homogeneous mode (500 µl flow cell).

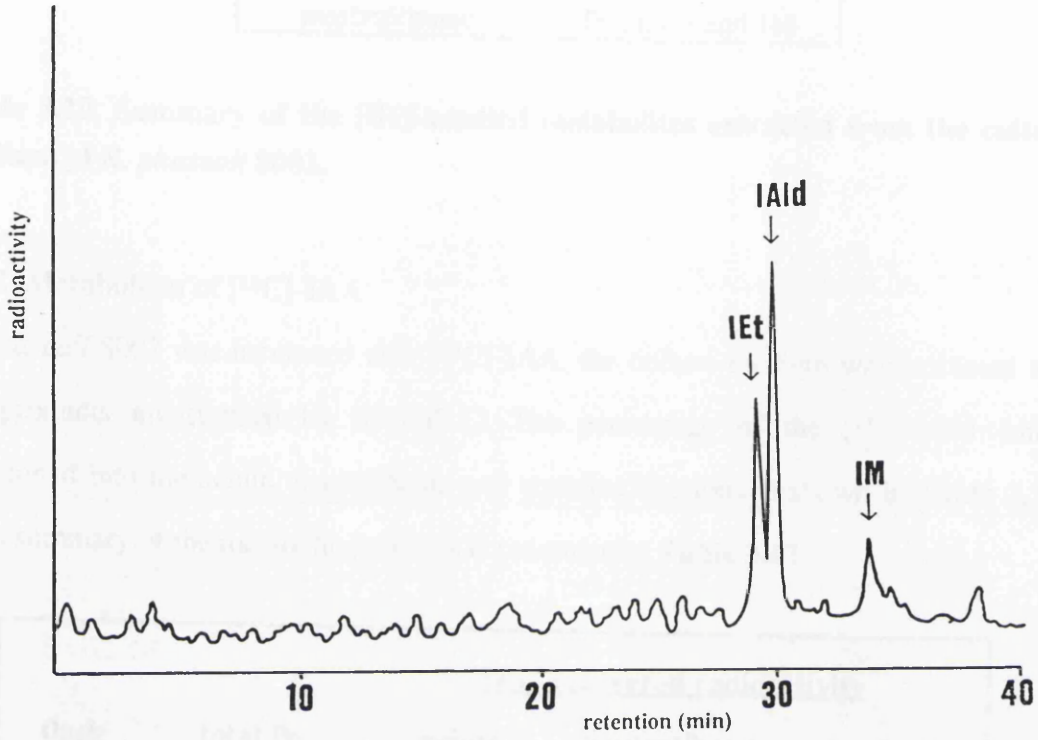


Fig. 3.5.4. Reversed-phase HPLC analysis of an aliquot (66 Kdpm) of neutral/basic ethyl acetate fraction obtained from the culture medium of *Rhizobium phaseoli* after a 3 h incubation with [^{3}H]-tryptophan (120 rpm, 27°C).

Column: 250 x 5 mm i.d., ODS Hypersil (5 µm). Mobile phase: methanol in 0.5% acetic acid (pH 6.5), 0-5 min 10%, 5-30 min 10-60%. Flow rate: 1 ml min^{-1}. Detector: Radioactivity monitor operating in the homogeneous mode (500 µl flow cell).

fraction	labelled metabolites
acidic	IAA, ILA
neutral/basic	IEt, IAld and IM

Table 3.10. Summary of the [^{3}H]-labelled metabolites extracted from the culture medium of *R. phaseoli* 8002.

3.6.2. Metabolism of [^{14}C]-IAA

R. phaseoli 8002 was incubated with [^{14}C]-IAA, the culture medium was extracted and the products investigated by RP-HPLC. The percentage of the [^{14}C]-label which partitioned into the acidic, neutral/basic and aqueous fractions is shown in **Table 3.11.** and a summary of the metabolic products is presented in **Table 3.12.**

flask	total % recovery	% of recovered radioactivity		
		acidic fraction	neutral/basic fraction	aqueous fraction
control	67	95.5	1.5	3
flask 1	78	82	16	2
flask 2	77	80	17.5	2.5

Table 3.11. Partitioning of [^{14}C]-labelled metabolites extracted from the culture medium of *R. phaseoli* 8002. The percentage of [^{14}C]-label which partitioned into the ethyl acetate acidic, neutral/basic and aqueous fractions after feeding *R. phaseoli* 8002 for 3 h with [^{14}C]-IAA. Cells were grown in defined minimal liquid media containing 5 mg l^{-1} tryptophan in an orbital shaker (120 rpm) at 27°C.

Over 90% of the label which was recovered was found in the acidic fraction of the control flask. This indicated that the procedure used for extraction of IAA was efficient. HPLC analysis of the control fractions revealed one peak, [^{14}C]-IAA in the acidic

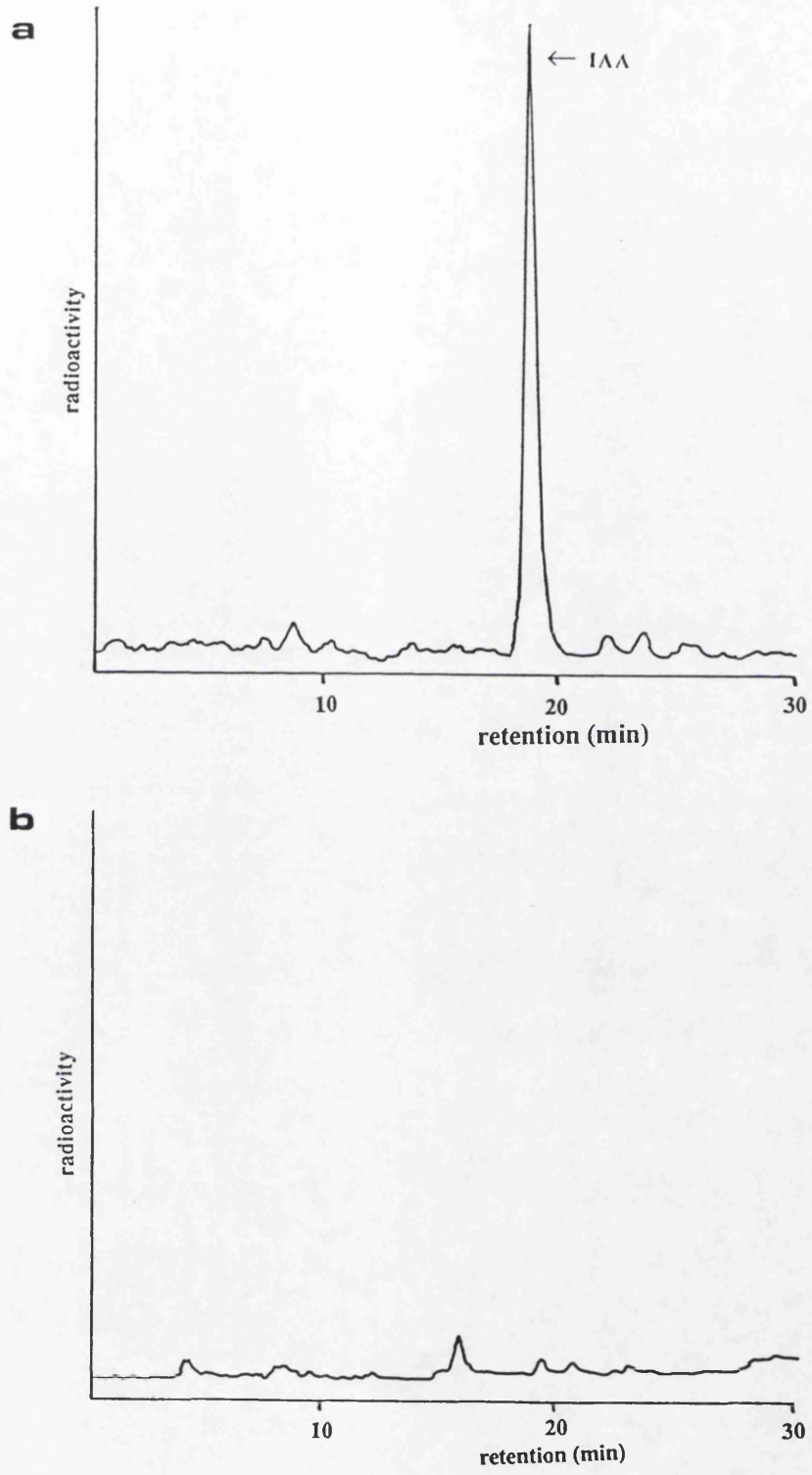


Fig. 3.6.1. Reversed-phase HPLC analysis of the (a) acidic fraction and (b) neutral/basic fraction obtained from the control (minus *Rhizobium*) flask after a 3 h incubation with [^{14}C]-IAA (120 rpm, 27°C).

Column: 250 x 5 mm i.d., ODS Hypersil (5 µm). Mobile phase: 30-70% methanol over 30 min in 1% acetic acid. Flow rate: 1 ml min^{-1}. Detector: Radioactivity monitor operating in the homogeneous mode (500 µl flow cell).

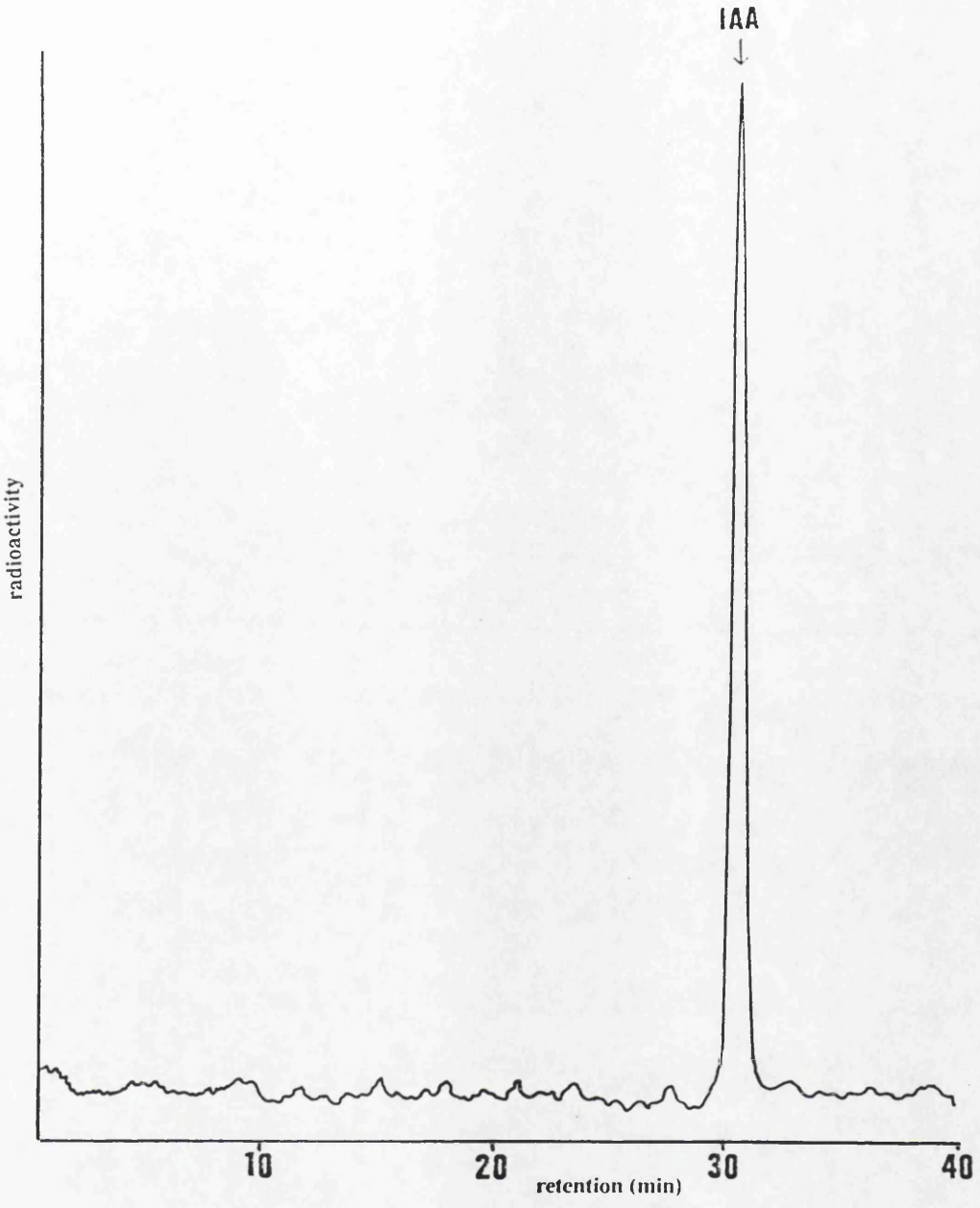


Fig. 3.6.2. Reversed-phase HPLC analysis of an aliquot (25 Kdpm) of acidic ethyl acetate fraction obtained from the culture medium of *Rhizobium phaseoli* after a 3 h incubation with [^{14}C]-IAA (120 rpm, 27°C).

Column: 250 x 5 mm i.d., ODS Hypersil (5 µm). Mobile phase: methanol in 1% acetic acid, 0-5 min 10%, 5-30 min 10-60%. Flow rate: 1 ml min^{-1}. Detector: Radioactivity monitor operating in the homogeneous mode (500 µl flow cell).

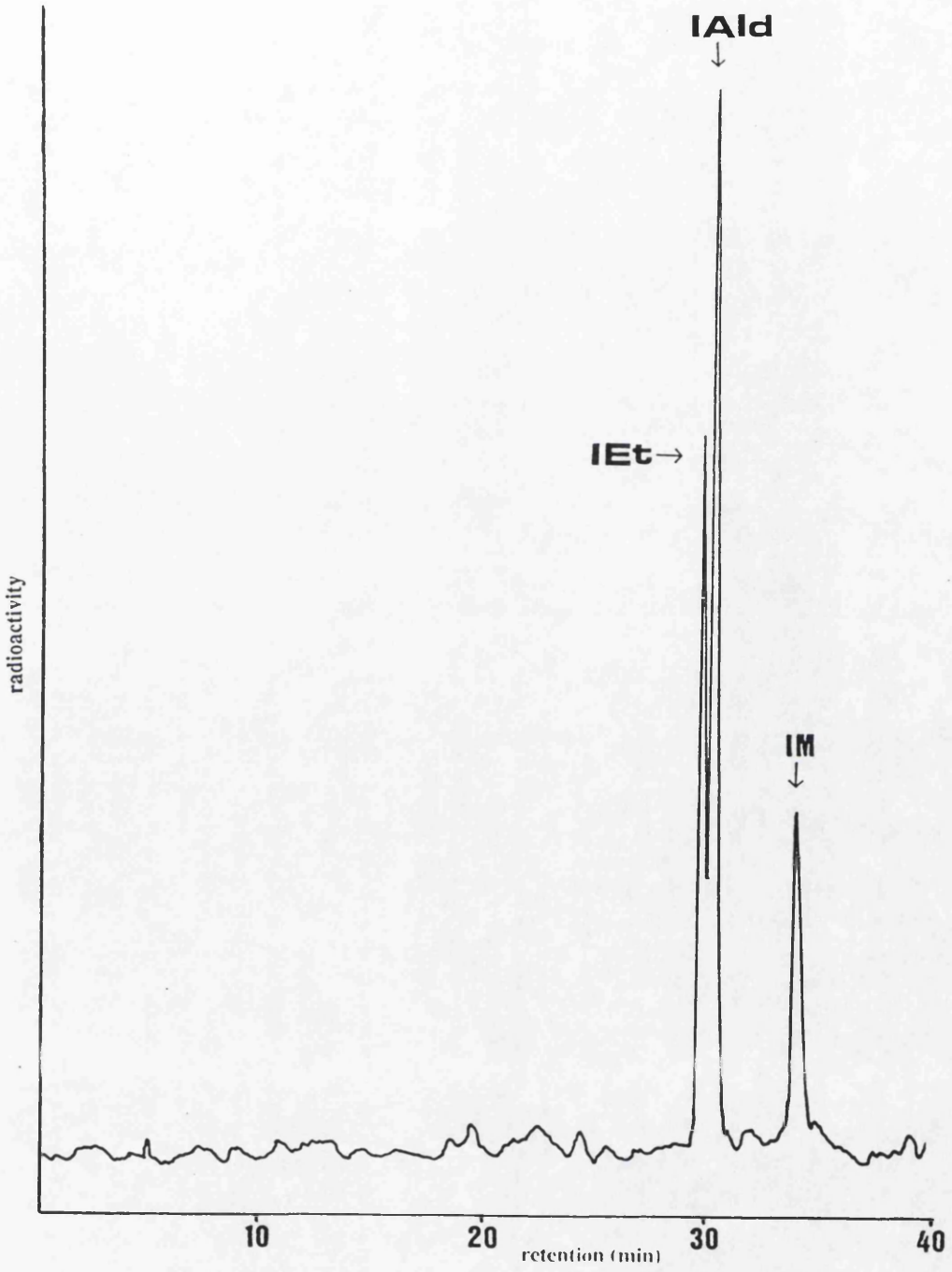


Fig. 3.6.3. Reversed-phase HPLC analysis of an aliquot (25 Kdpm) of neutral/basic ethyl acetate fraction obtained from the culture medium of *Rhizobium phaseoli* after a 3 h incubation with [^{14}C]-IAA (120 rpm, 27°C).

Column: 250 x 5 mm i.d., ODS Hypersil (5 µm). Mobile phase: methanol in 0.5% acetic acid (pH 6.5), 0-5 min 10%, 5-30 min 10-60%. Flow rate: 1 ml min^{-1}. Detector: Radioactivity monitor operating in the homogeneous mode (500 µl flow cell).

fraction (**Fig. 3.6.1a.**), whereas in the neutral/basic fraction no peaks were found (**Fig. 3.6.1b.**) indicating that the IAA had not broken down into any acidic or neutral/basic products.

For each of the experimental flasks slightly more than 3/4 of the radioactivity added was recovered and of this, around 80% partitioned into the acidic ethyl acetate fraction. RP-HPLC analysis of the acidic fraction (**Fig. 3.6.2.**) revealed only one radioactive peak which had a retention time similar to that of IAA (**Fig. 3.5.3.**). This peak appeared therefore to be unmetabolised [^{14}C]-IAA.

Slightly less than a fifth of the label partitioned into the neutral/basic fraction of the experimental flasks. RP-HPLC of this fraction revealed three radioactive peaks (**Fig. 3.6.3.**) which had similar retention times to authentic IEt, IAld and IM.

fraction	labelled metabolites
acidic	IAA (label)
neutral/basic	IEt, IAld and IM

Table 3.12. Summary of the [^{14}C]-labelled metabolites extracted from the culture medium of *R. phaseoli* 8002.

3.7. Metabolism of [^{3}H]-tryptophan and [^{14}C]-IAA by detached root and nodule tissue of *Phaseolus vulgaris*

3.7.1. Metabolism of [^{3}H]-tryptophan

The metabolism of [^{3}H]-tryptophan by detached root and nodule tissue of *P. vulgaris* was investigated (**2.4.**). The resulting labelled metabolites were analysed by RP-HPLC and the results are summarised in **Table 3.13.**

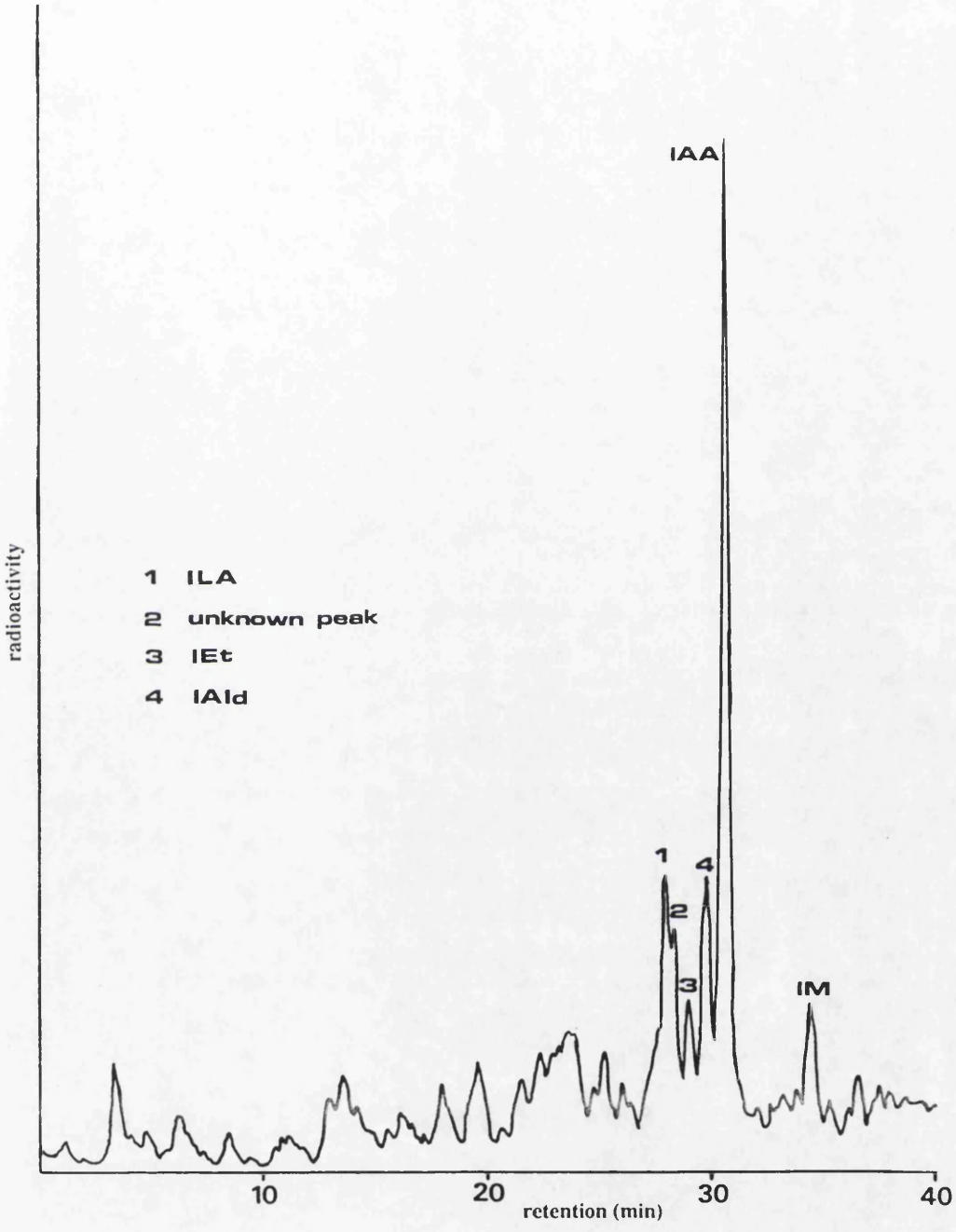


Fig. 3.7.1. Reversed-phase HPLC analysis of a 100 Kdpm methanolic extract obtained from detached nodule tissue of *Phaseolus vulgaris* after 12 h incubation with [^{3}H]-tryptophan.

Column: 250 x 5 mm i.d., ODS Hypersil (5 µm). Mobile phase: methanol in 1% acetic acid, 0-5 min 10%, 5-30 min 10-60%. Flow rate: 1 ml min^{-1}. Detector: Radioactivity monitor operating in the homogeneous mode (500 µl flow cell).

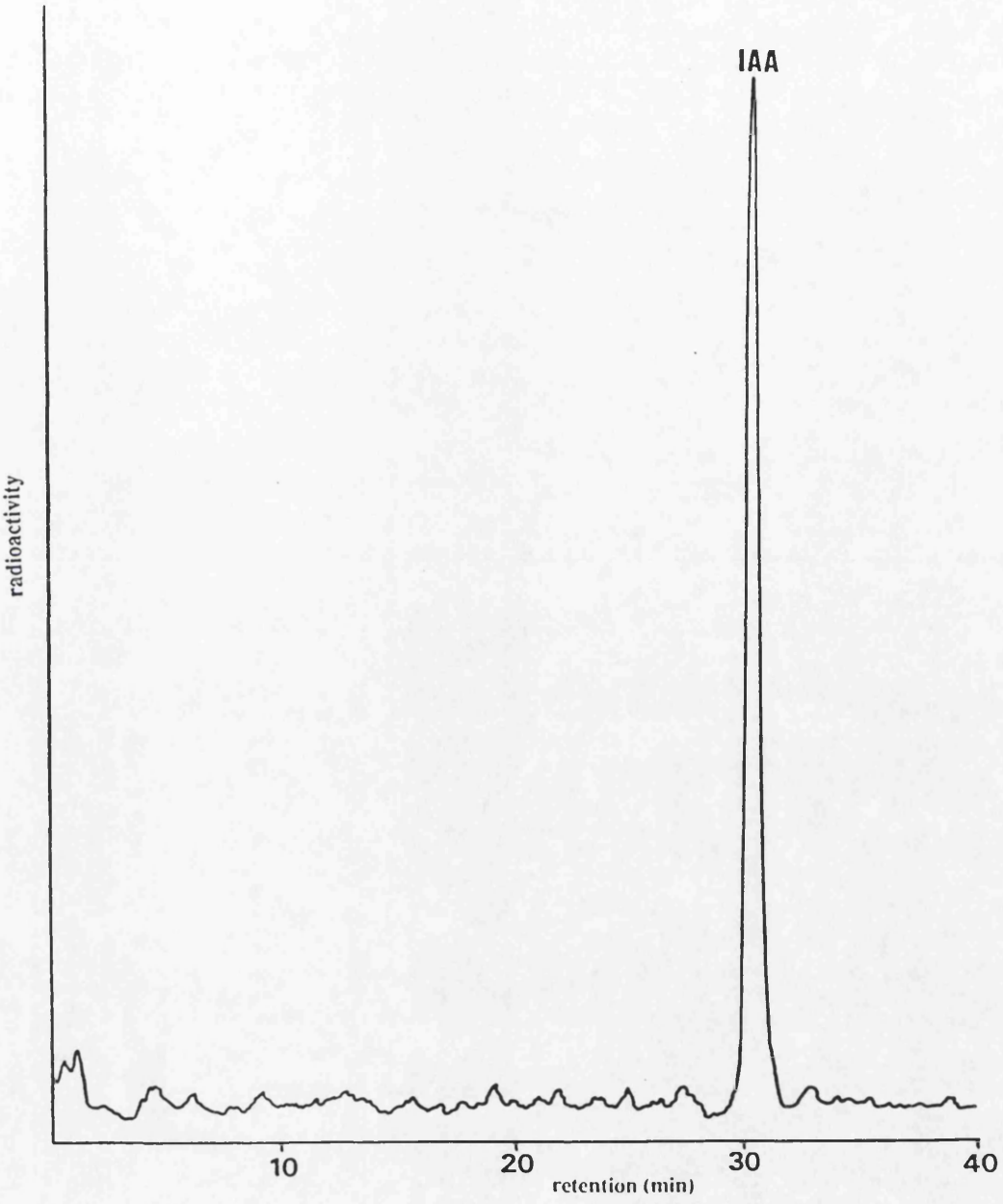


Fig. 3.7.2. Retention time of [^{14}C]-IAA standard (50 Kdpm) on reversed-phase HPLC.

Column: 250 x 5 mm i.d., ODS Hypersil (5 µm). Mobile phase: methanol in 1% acetic acid, 0-5 min 10%, 5-30 min 10-60%. Flow rate: 1 ml min^{-1}. Detector: Radioactivity monitor operating in the homogeneous mode (500 µl flow cell).

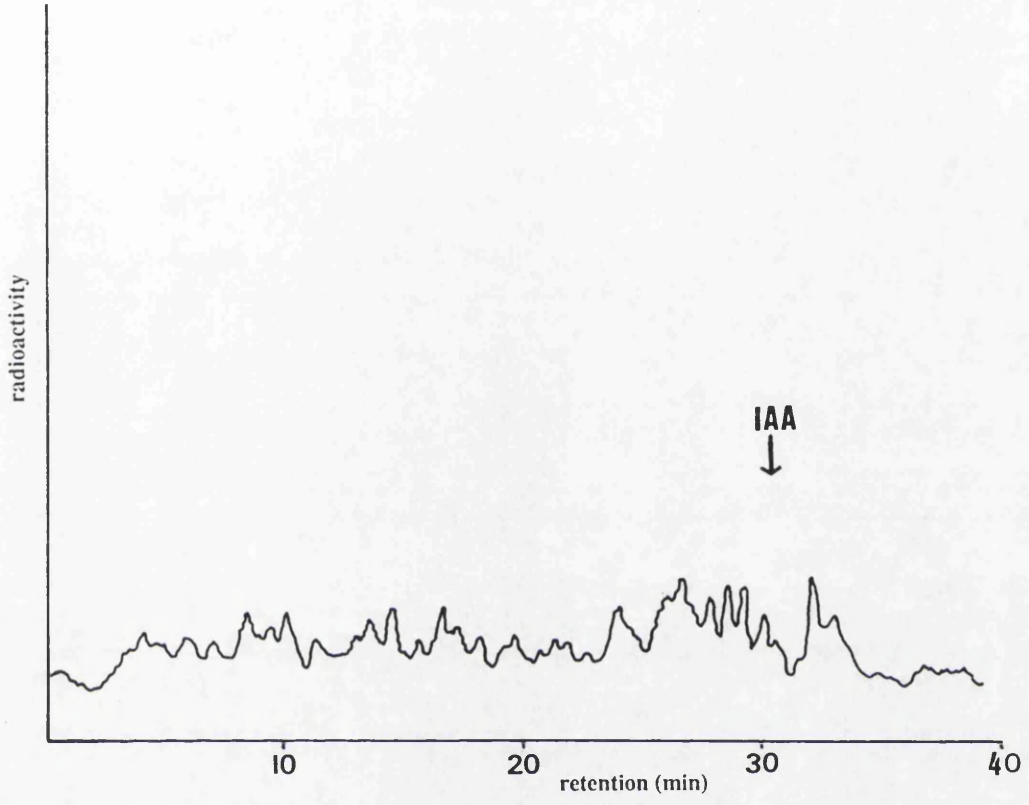


Fig. 3.7.3. Reversed-phase HPLC analysis of a 100 Kdpm methanolic extract obtained from detached root tissue of *Phaseolus vulgaris* after 12 h incubation with [^{3}H]-tryptophan. Column: 250 x 5 mm i.d., ODS Hypersil (5 µm). Mobile phase: Methanol in 1% acetic acid, 0-5 min 10%, 5-30 min 10-60%. Flow rate: 1 ml min^{-1}. Detector: Radioactivity monitor operating in the homogeneous mode (500 µl flow cell). Retention time of IAA (30.5 min) is indicated.

The feeding of [^{3}H]-tryptophan to nodule tissue resulted in the formation of one major and five smaller radioactive peaks (**Fig. 3.7.1.**). The largest peak had a retention time similar to that of IAA (**Fig. 3.7.2.**) and four of the smaller peaks had similar retention times to ILA, IEt, IAld and IM. Peak 2 remained unidentified although it had a similar retention time to indole carboxylic acid (ICA) (**Fig. 3.2.**). It was not possible however, to verify this with co-chromatography using the fluorescence monitor, as the sample contained a large number of fluorescent compounds.

In contrast to the large number of radiolabelled indoles extracted from nodule tissue, when [^{3}H]-tryptophan was fed to detached roots for 6 and 12 h, no major radioactive peaks were detected (**Fig. 3.7.3.**). It appeared that in root tissue there was no conversion of the labelled tryptophan to IAA or to any other indolic compound.

Tissue	Incubation time	Labelled products
root	6 h	no indolic metabolites
root	12 h	no indolic metabolites
nodule	12 h	ILA, IAA, IEt, IAld, IM and (ICA?)

Table 3.13. Summary of the HPLC analysis of the metabolism of [^{3}H]-tryptophan by detached root and nodule tissue of *P. vulgaris*. Detached roots and nodules of *P. vulgaris* were incubated for 6 or 12 h with [^{3}H]-tryptophan in phosphate buffer, KH_2PO_4 (1 mM, adjusted to pH 7.0 with NaOH) in darkness at 25°C.

3.7.2. Metabolism of [^{14}C]-IAA

IAA metabolism by *P. vulgaris* detached root and nodule tissue was investigated with [^{14}C]-labelled IAA feeds. The analysis of the resulting labelled metabolites by RP-HPLC is shown in **Figs. 3.8.1. - 3.8.3.** and summarised in **Table 3.14.**

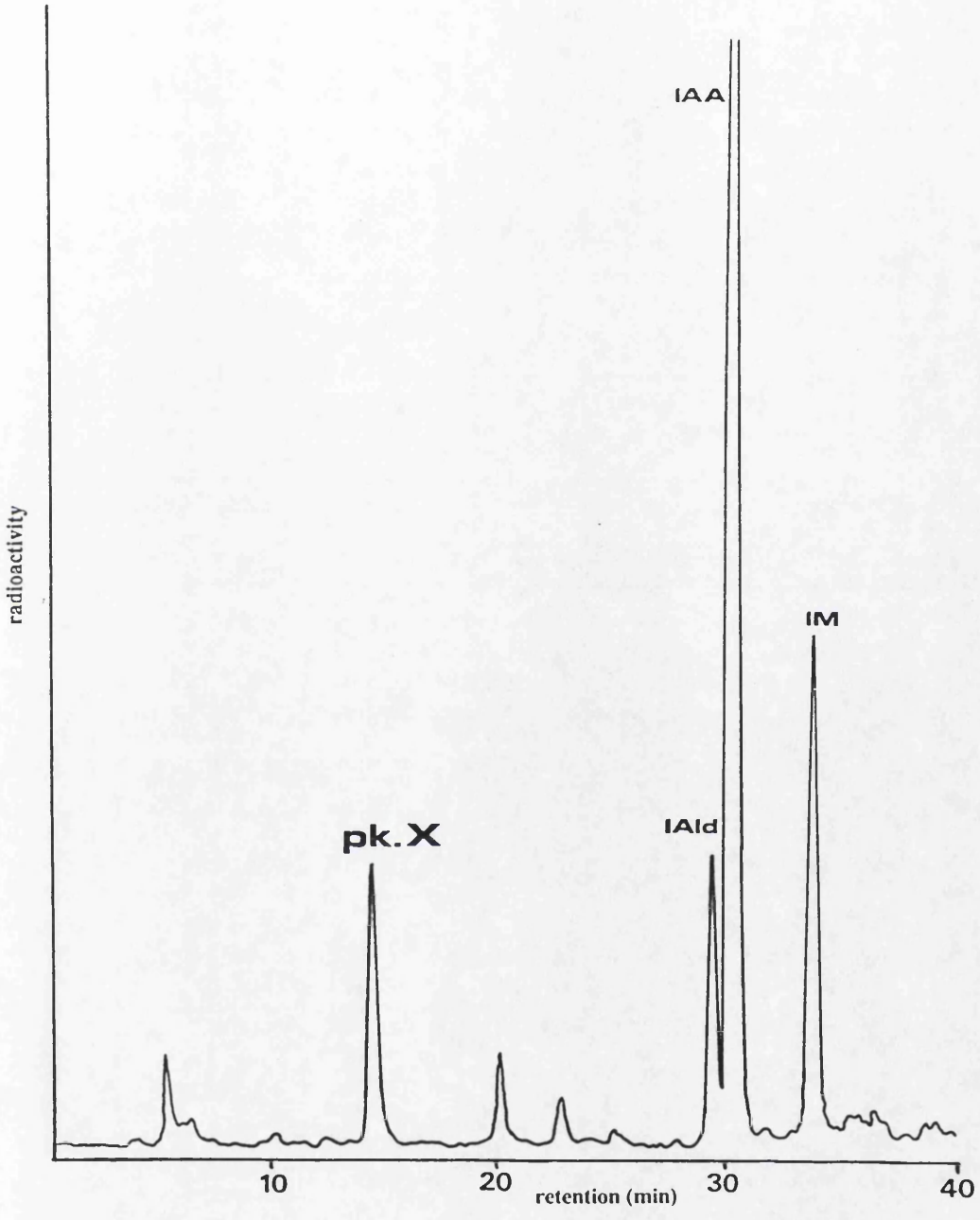


Fig. 3.8.1. Reversed-phase HPLC analysis of a 100 Kdpm methanolic extract obtained from detached nodule tissue of *Phaseolus vulgaris* after 24 h incubation with [^{14}C]-IAA.

Column: 250 x 5 mm i.d., ODS Hypersil (5 µm). Mobile phase: methanol in 1% acetic acid, 0-5 min 10%, 5-30 min 10-60%. Flow rate: 1 ml min^{-1}. Detector: Radioactivity monitor operating in the homogeneous mode (500 µl flow cell).

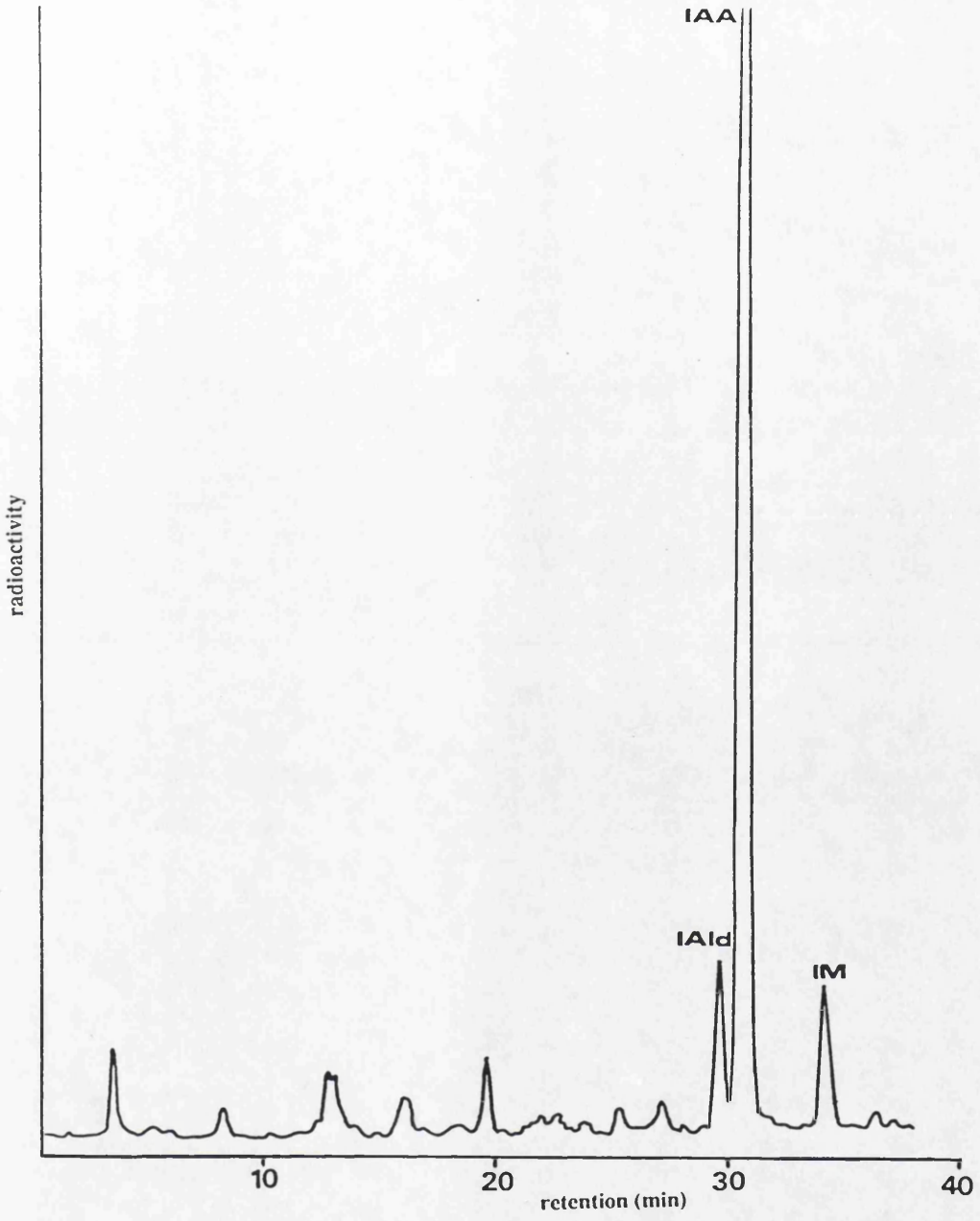


Fig. 3.8.2. Reversed-phase HPLC analysis of a 100 Kdpm methanolic extract obtained from detached root tissue of *Phaseolus vulgaris* after 12 h incubation with [^{14}C]-IAA.

Column: 250 x 5 mm i.d., ODS Hypersil (5 µm). Mobile phase: methanol in 1% acetic acid, 0-5 min 10%, 5-30 min 10-60%. Flow rate: 1 ml min^{-1}. Detector: Radioactivity monitor operating in the homogeneous mode (500 µl flow cell).

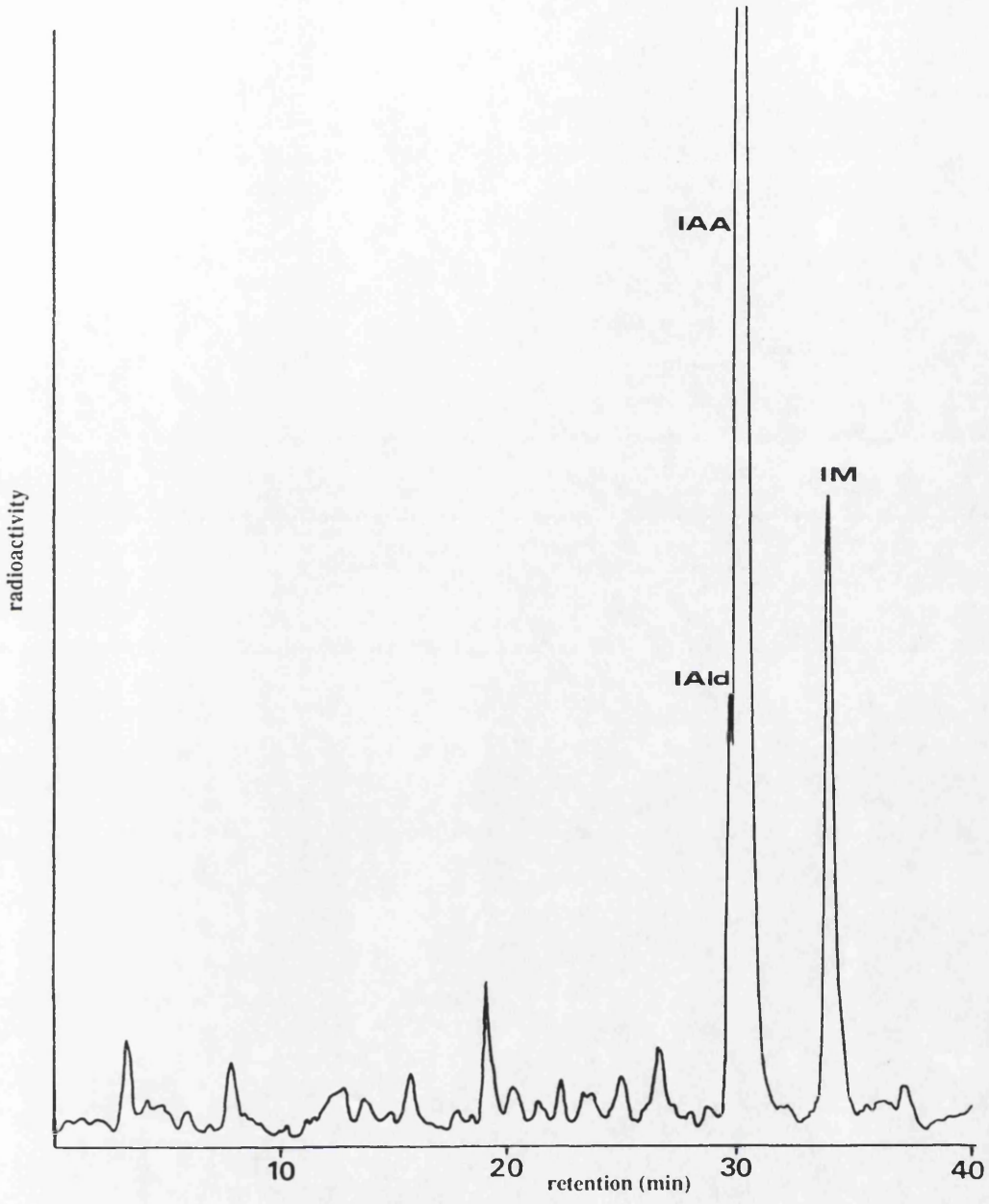


Fig. 3.8.3. Reversed-phase HPLC co-chromatography of methanolic extracts of root and nodule tissue of *Phaseolus vulgaris* (25 Kdpm of each) after incubation with [^{14}C]-IAA for 12 and 24 h respectively. Column: 250 x 5 mm i.d., ODS Hypersil (5 µm). Mobile phase: methanol in 1% acetic acid, 0-5 min 10%, 5-30 min 10-60%. Flow rate: 1 ml min^{-1}. Detector: Radioactivity monitor operating in the homogeneous mode (500 µl flow cell).

Analysis of the [^{14}C]-IAA feed to detached nodule tissue for 6 and 12 h revealed that metabolism of the label had not occurred as only IAA was present. However, with longer incubation (24 h), three major radioactive peaks were detected in addition to IAA. Two of these peaks had retention times similar to IAld and IM (**Fig. 3.8.1.**). However, the identity of the peak with the retention time of (14 min) (Peak X) is unknown. The early elution of this peak suggests that it is a breakdown product of IAA rather than a true metabolite.

When [^{14}C]-IAA was fed to detached root tissue for 6 and 12 h (**Fig. 3.8.2.**), the majority of the activity was found in the large unmetabolised IAA peak. There were, however, 2 smaller peaks with retention times similar to IAld and IM.

When both root and nodule extracts were combined and analysed by RP-HPLC, the IAA, IAld and IM peaks co-chromatographed with each other indicating an identical spectrum of [^{14}C]-IAA metabolites in the root and nodule tissue (**Fig. 3.8.3.**).

Tissue	Incubation time	Labelled metabolites
root	6 h	IAld, IM
root	12 h	IAld, IM
nodule	6 h	no indolic metabolites
nodule	12 h	no indolic metabolites
nodule	24 h	IAld, IM

Table 3.14. Summary of the HPLC analysis of the metabolism of [^{14}C]-IAA by detached root and nodule of *P. vulgaris*. Detached roots and nodules of *P. vulgaris* were incubated for 6, 12 or 24 h with [^{14}C]-IAA in phosphate buffer, KH_2PO_4 (1 mM, adjusted to pH 7.0 with NaOH) in darkness at 25°C.

3.8. Conclusions

The work reported in this chapter describes the production of indolic compounds by *Rhizobium phaseoli* 8002 and its host plant, *Phaseolus vulgaris*.

Reversed and normal phase HPLC co-chromatography with standards and the mass spectra of these peaks, suggest that the major indolic compounds which accumulate in both tryptophan (+) and (-) medium of *Rhizobium* are IAA, ILA, IEt, IAld and IM. The addition of tryptophan to the medium stimulated the production of all indoles, although the extent of the increase varied for each compound.

An investigation into possible synergism between IAA and gibberellic acid (10^{-5} and 10^{-6} M GA_3) in *R. phaseoli* indicated an increase in production at low GA_3 concentrations, however this effect was not reproducible in subsequent experiments.

In metabolism studies, *R. phaseoli* was shown to convert [^{3}H]-tryptophan to IAA, ILA, IEt, IAld and IM, and [^{14}C]-IAA to IEt, IAld and IM. Thus the spectrum of metabolites produced by metabolism of tryptophan and IAA is identical to those indoles detected in the rhizobial culture medium.

Radiolabelled feeds to detached root and nodule tissue of *Phaseolus vulgaris* resulted in the conversion of [^{3}H]-tryptophan to products with retention times similar to IAA, ILA, IEt, IAld and IM in nodule tissue, while in root tissue no indolic metabolites were detected. The addition of [^{14}C]-IAA resulted in the production of metabolites with retention times similar to IAld and IM in both root and nodule tissue. The spectrum of metabolites produced by the metabolism of tryptophan and IAA by nodule tissue is therefore identical to those produced by the bacterium *in vitro* and hence it was not

possible to discriminate between host and bacterial IAA production in the symbiotic state.

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possible to discriminate between host and bacterial IAA production in the symbiotic state.

CHAPTER 4

RESULTS

4.1. Transposon mutagenesis of *Rhizobium phaseoli*

4.1.1. Aims

The objective of the research described in this chapter was to create IAA minus mutants or IAA under or over producers of *R. phaseoli* using Tn5 mutagenesis. As the transposon carries a gene conferring resistance to neomycin (which was to be used as a selectable marker) the intrinsic susceptibility of two strains of *Rhizobium* to the antibiotic was evaluated as a prerequisite to mutagenesis. After conjugal mating, the neomycin resistant transconjugants were screened for the production of IAA using Salkowski reagent.

4.1.2. Susceptibility of *Rhizobium phaseoli* strain 4292 to rifampicin

It was hoped that *Rhizobium phaseoli* 4292 (John Innes Institute, Norwich), derived from strain 8002, reported to exhibit a rifampicin resistant phenotype, could be used as the recipient for the Tn5 transposon. Rifampicin would thus act as an extra selection against *E. coli* cells (plasmid donor), in addition to minimal medium plates on which *E. coli* are unable to survive.

When strain 4292 was plated onto medium containing rifampicin at concentrations between 0.2 and 200 $\mu g\ ml^{-1}$, colonies formed only on rifampicin (-) plates. The concentration of rifampicin was decreased to between 0.2 and 200 $ng\ ml^{-1}$ and **Fig. 4.1a.** illustrates the percentage survival of strain 4292 grown on rifampicin at these concentrations. The level of rifampicin resistance found in strain 4292 was similar to that of strain 8002 (**Fig. 4.1b.**). Consequently, as 4292 did not offer any benefit for mutagenesis, the latter strain was used as the recipient for Tn 5.

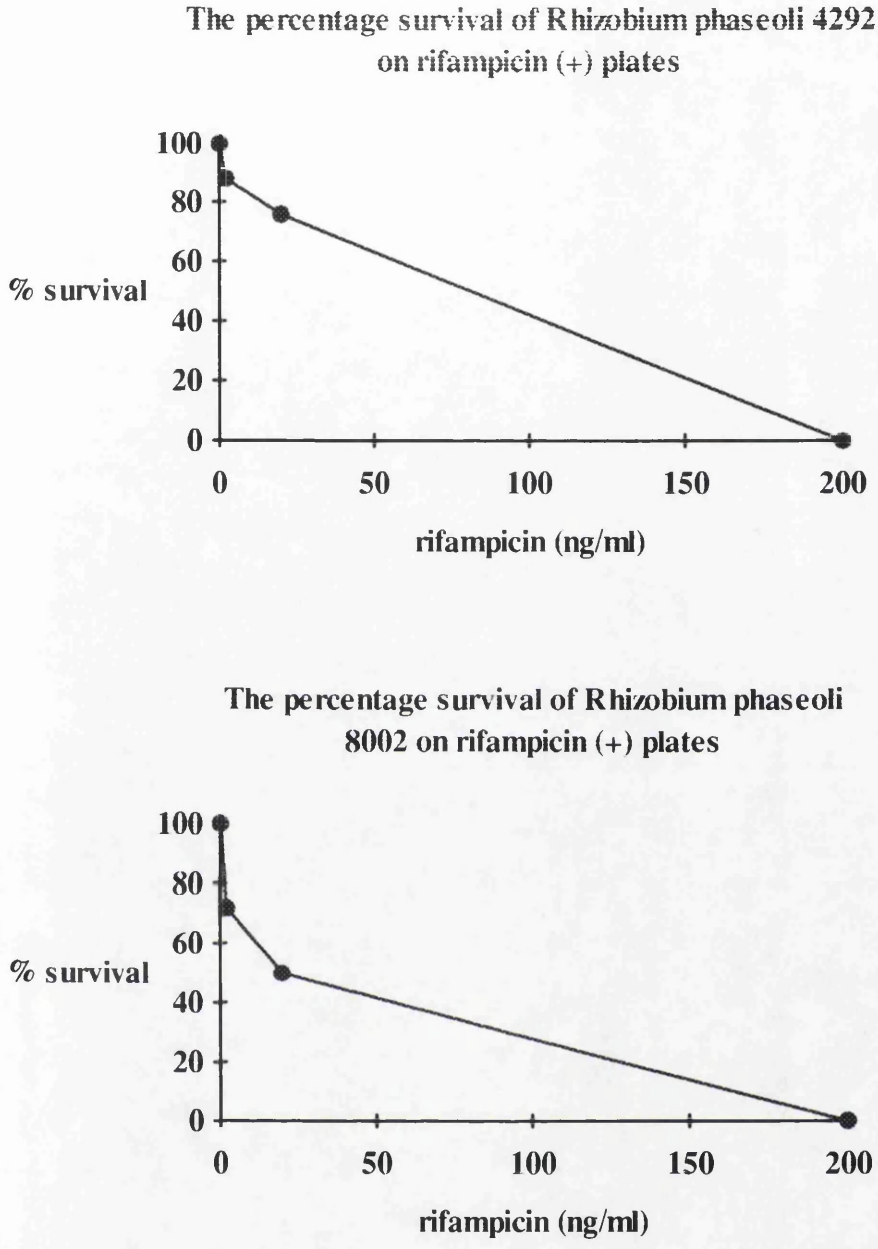


Fig. 4.1. The percentage survival of cells of *R. phaseoli* **(a)** strain 4292 and **(b)** strain 8002 growing on solid medium containing rifampicin.

4.1.3. Survival of *R. phaseoli* 8002 on neomycin (+) plates

R. phaseoli strain 8002 was plated onto neomycin at concentrations between 0 and 50 μg ml^{-1} in order to determine an effective concentration to select against untransposed cells i.e. those without the transposon-borne resistance gene. **Fig. 4.2.** and **Fig. 4.3.**

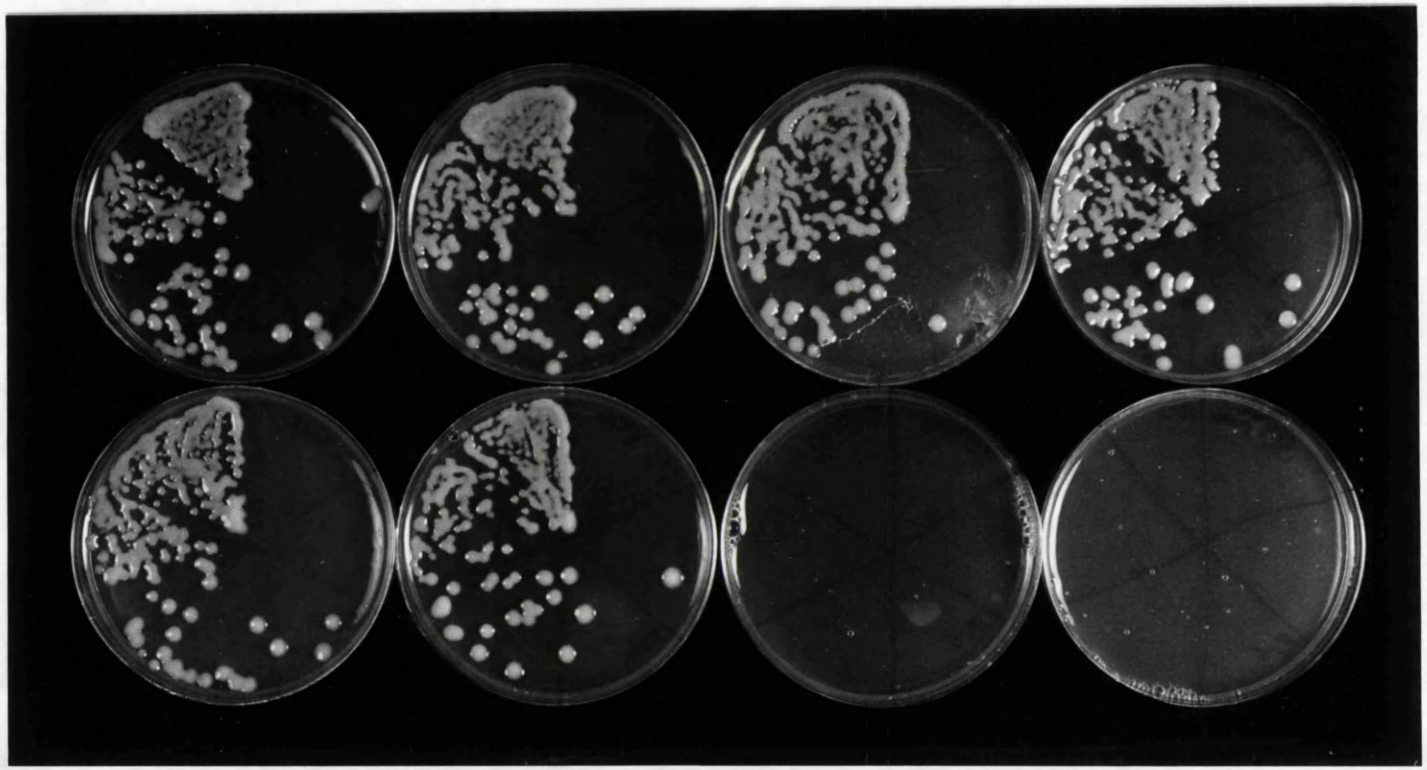


Fig. 4.2. The effect of the antibiotic neomycin on the survival of *R. phaseoli* strain 8002. Top row (left to right): plates contain 0, 0.1, 1, 2 μg ml^{-1} neomycin. Bottom row (left to right):plates contain 5, 10, 20, 50 μg ml^{-1} neomycin. Each plate was divided into six segments and serial dilutions between 10^{-3} and 10^{-8} bacteria plated on each.

show the susceptibility of cells to concentrations of 20 µg ml^{-1} neomycin and over. A concentration of 30 µg ml^{-1} was therefore chosen to select against untransposed *Rhizobium*.

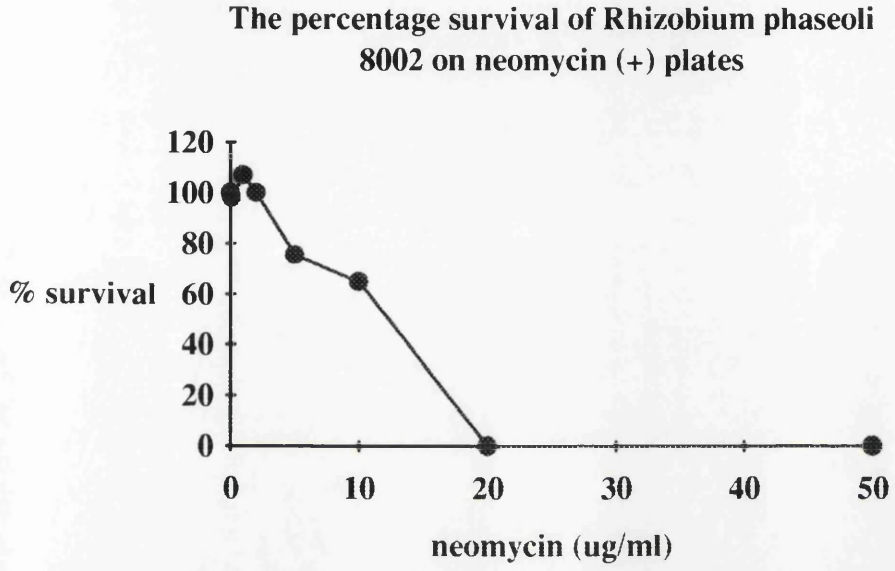


Fig. 4.3. The percentage survival of cells of *R. phaseoli* strain 8002 growing on solid medium containing neomycin.

4.1.4. Transposon mutagenesis of *R. phaseoli* strain 8002 using di-parental mating

Di-parental matings were carried out initially to establish the applicability of this technique i.e. whether transposon Tn5 could be transferred to *Rhizobium* and, if so to determine the optimum ratio of donor to recipient cells for subsequent matings.

E. coli strain 17-1[2021] (donor) containing the mobilisable 'suicide' plasmid pSUP2021 was mixed with *R. phaseoli* 8002 (recipient) in the ratios 1:1, 5:1, 10:1, 50:1, 100:1 and 200:1 *Rhizobium* to *E. coli* cells. After mating the cells were resuspended and plated, as described in **2.7.5. Figs. 4.4a.** and **b** show that cells which have integrated the Tn5 after mating become neomycin resistant and are able to form colonies, while those which have not been mated and have not received the transposon have no such resistance and fail to

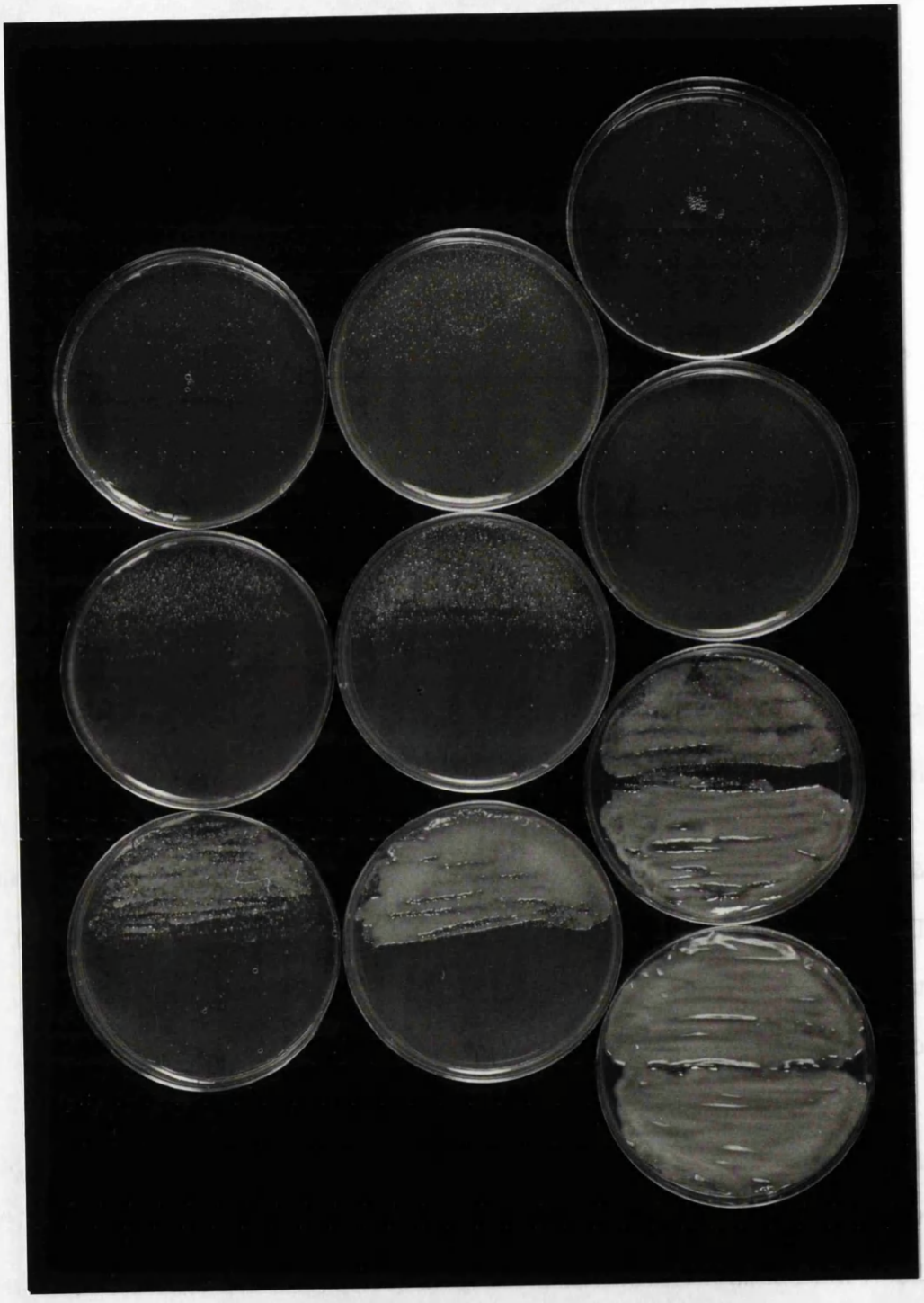


Fig. 4.4a. Diparental mating between *E. coli* 17-1[2021] and *R. phaseoli* strain 8002.

Row A: Mated and unmated *Rhizobium* cells were plated on the right and left side of neomycin plus (30 μg ml^{-1}) plates respectively, at dilutions 10°, 10^{-1} and 10^{-2} from left to right. *Rhizobium* were mated with *E. coli* in the ratio of 1:1. Only cells which have acquired the transposon during mating are able to survive on the antibiotic plates. **Row B**: As above except *Rhizobium* cells were mated with *E. coli* at 10:1. The *Rhizobium/E. coli* mixtures were mated for 6 h at 37°C before harvesting and selecting on neomycin medium. The methods are described in detail in **2.7.5. Row C**: Neomycin (+) and (-) control plates were plated with unmated *Rhizobium* at dilutions between 10^{0} and 10^{-3}. The pair on the left are neomycin (-) and so the cells are able to survive while the pair on the right are neomycin (+) and as a consequence all the cells are killed.

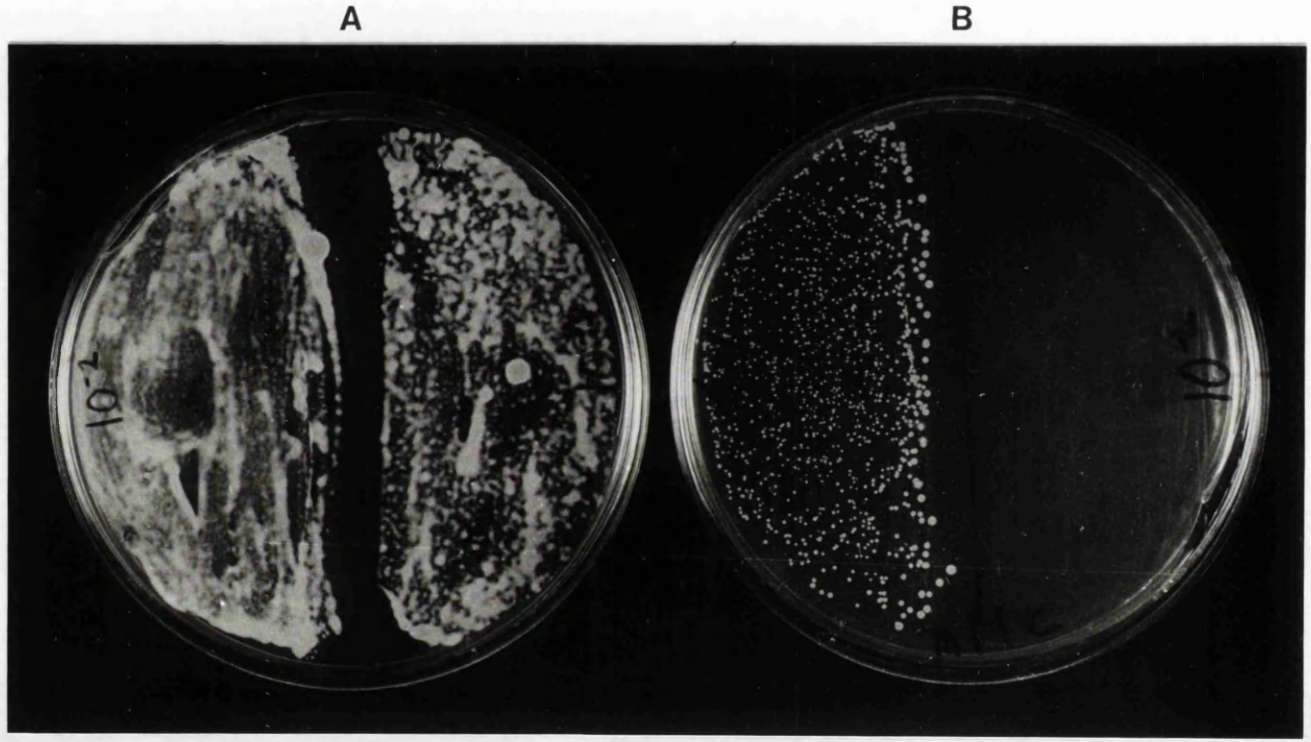


Fig. 4.4b. Diparental mating between *E. coli* 17-1[2021] and *R. phaseoli* strain 8002.

Plate A: Unmated *Rhizobium* plated on neomycin (-) control plate at dilutions of 10^{-2} (left side) and 10^{-3} (right side). **Plate B**: Mated and unmated *Rhizobium* plated on neomycin (+) medium (30 µg ml^{-1}) on the left and right respectively. The *Rhizobium*/*E. coli* cells were mixed 1:1 and mated for 6 h at 37°C before harvesting and selecting on neomycin medium. The methods are described in detail in **2.7.5.**

survive on antibiotic. Additionally, cells were plated on neomycin (+) and (-) control plates to demonstrate respectively, that the antibiotic was lethal and that the *Rhizobium* cells were viable before mating had begun.

The frequency of neomycin resistant (Nm^r) colonies after conjugation (**Table 4.1.**) is a function of the frequency of transfer and the frequency of transposition, the former being more efficient than the latter (Hunter, C. N., 1988).

Ratio *Rhizobium* : *E. coli*	Frequency of Nm^r transconjugants
1:1	2.2×10^{-5}
5:1	5.5×10^{-5}
10:1	6.7×10^{-5}
50:1	6.2×10^{-5}
100:1	4.8×10^{-5}
200:1	4.1×10^{-5}

Table 4.1. Di-parental mating between *E. coli* 17-1[2021] and *Rhizobium phaseoli* 8002. The frequency of Nm^r transconjugants resulting from di-parental matings between *R. phaseoli* 8002 and *E. coli* 17-1[2021] cells mixed at the above ratios. The mixture was placed onto a well-dried plate of LB-agar and incubated for 6 h at 37°C after which they were harvested and plated on neomycin (30 µg ml^{-1}) medium to select for transconjugants. The methods are described in detail in **2.7.5.** The transfer frequencies are given per number of recipient cells e.g. at ratio 1:1 for every 10^6 recipient cells that underwent mating 22 acquired neomycin resistance.

The frequency of Nm^r transconjugants resulting from the random insertion of Tn5 following conjugative transfer of pSUP2021 to strain 8002 was greater than that determined in the photosynthetic bacterium *Rhodobacter sphaeroides* which was approximately 2×10^{-6} per recipient (Hunter, C. N., 1988). However, the frequency of Nm^r transconjugants arising from the random insertion of the transposon into *Rhizobium meliloti* is of a similar order of magnitude at 10^{-5} per recipient (Simon *et al.*, 1983).

As a result of these preliminary matings, the most efficient ratio of *Rhizobium* to *E. coli* cells (10:1) was chosen for the creation of transconjugants to be tested for IAA production.

4.2. Screening mutant colonies for IAA production

4.2.1. Reaction of Salkowski reagent with IAA

Increasing amounts of IAA (0-10 µg), added to 100 µl of Salkowski reagent (Gordon and Weber, 1951; **2.8.1.**), produced a range of colour from colourless to dark red; the intensity of colour increasing with the amount of IAA present in the sample. **Fig. 4.5a.** shows the reaction of Salkowski reagent with 0, 50, 100, 250 and 500 ng IAA and **Fig. 4.5b.**, the reaction with 750 ng and 1, 2.5, 5 and 10 µg of IAA. The colour produced in the reaction between IAA and the reagent was stable up to 24 h.

4.2.2. Reaction of Salkowski reagent with IAA produced on solid differential medium by *Rhizobium*

Rhizobium converts the high level of tryptophan (200 mg l^{-1}) in the differential medium to IAA and when tested with Salkowski reagent a pink colour develops within 2 min (in 3 day old colonies). Control plates, containing tryptophan but minus the bacteria were tested with Salkowski reagent. However as no colour was produced it was clear that the reagent did not react with tryptophan or other chemicals in the plates.

There were a number of problems associated with the detection of IAA on agar plates using the Salkowski reagent. Firstly, it was found that the reagent killed the cells, and secondly, IAA diffusing from a colony could interfere with the results obtained for nearby colonies. Both of these problems are discussed in further detail below in **(a)** and

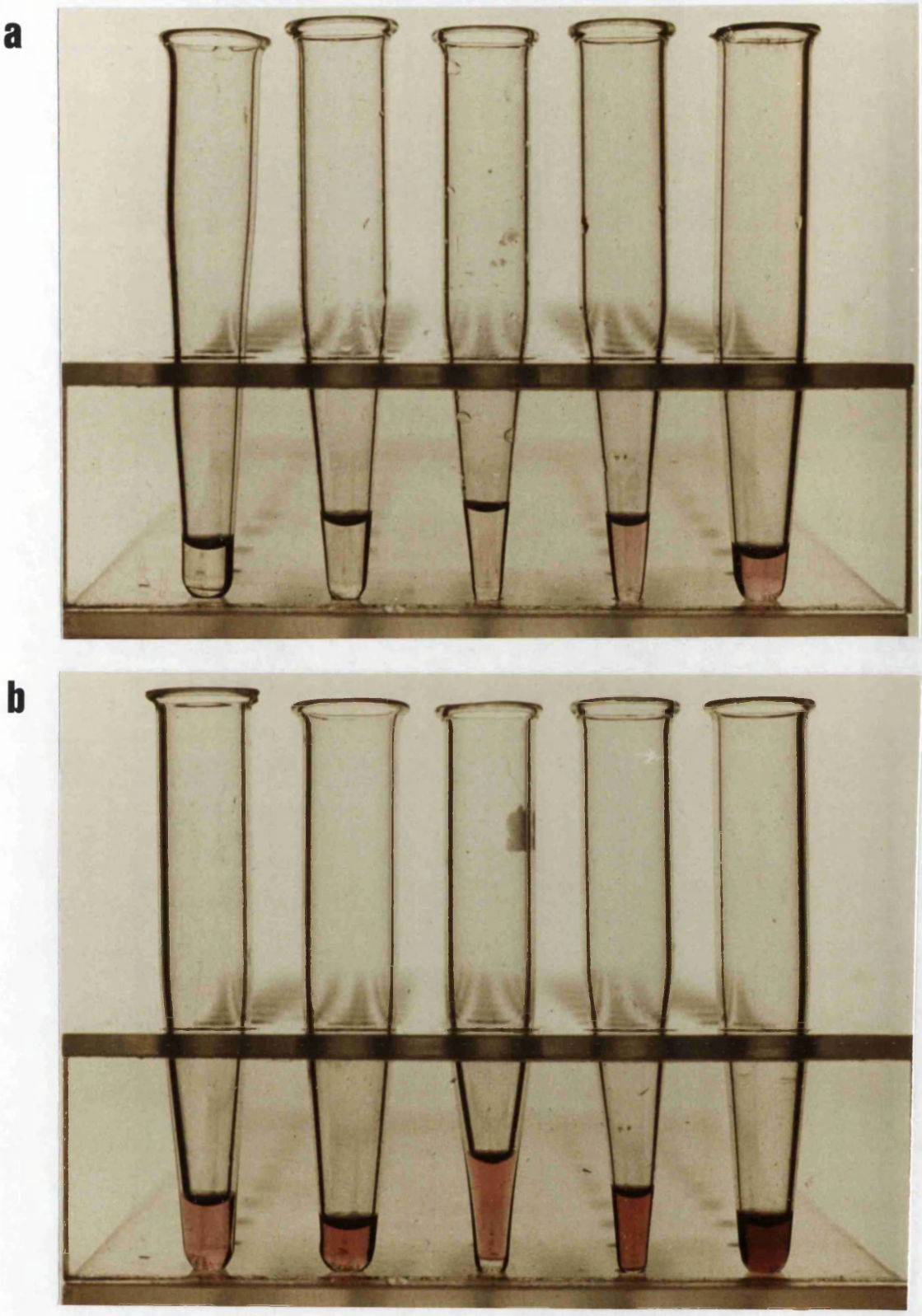


Fig. 4.5. The colour change associated with the reaction of Salkowski reagent with IAA. The reaction of 100 µl of Salkowski reagent with **(a)** 0, 50, 100, 250 and 500 ng, **(b)** 750 ng, 1, 2.5, 5 and 10 µg of indole-3-acetic acid (**2.8.1.**).

(b). In addition, as the reagent killed the cells the colonies had to be replicated. However replica plating of the colonies proved difficult as they were moist and tended to spread when the filter was placed on them.

All of these problems were overcome by taking a sample of cells and the underlying agar from the middle of the colony, where interference from other colonies was least likely, and placing this core in an Eppendorf tube for testing, instead of spotting the reagent directly onto the colony. Moreover, it was easier to see the colour of the reaction when the sample of agar was taken than when the reagent was simply spotted onto the plate. The amount of agar tested was standardised by using the same inoculating loop for each sampling and by having the same depth of agar in each plate.

(a) Diffusion of IAA from colonies on solid differential medium

Plates, spotted with the reagent, revealed that there was considerable diffusion away from the site of production. It was found that 4 day old colonies had to be on average, 2 cm away from the centre of the nearest colony to avoid interference from IAA production by that colony.

(b) The viability of *Rhizobium* colonies after spotting with Salkowski reagent

The viability of cells from colonies, 5 and 10 mm in diameter, after the addition of Salkowski reagent was determined by spotting the reagent at one side of the colony and removing cells from the opposite side at various intervals up to an hour (**Table 4.2.**). The ability of these cells to form colonies when re-plated onto fresh medium was taken as a measure of viability.

Time after addition (min)	Colonies 5 mm i.d.	10 mm i.d.
1	+	+
5	+	+
10	+/-	+
15	-	+
20	-	+
30	-	+
45	-	+
60	-	+/-
12 h	-	-

+ viable cells - inviable cells

Table 4.2. The viability of cells removed from *R. phaseoli* 8002 colonies after the addition of Salkowski reagent. The viability of cells removed from colonies 5 and 10 mm in diameter (3 and 4 days old respectively), after the addition of 3 drops of Salkowski reagent at the side of the colony. Cells, removed from the opposite side of the colony by a sterile loop were then re-plated onto fresh agar medium and grown at 28°C.

Cells removed from colonies 5 mm in diameter were rendered inviable 10 min after the addition of the reagent. In contrast, cells removed from colonies 10 mm in diameter remained viable up to 45 min, although only some of these colonies were viable at 60 min. The bacteria which had been in contact with the reagent appeared to 'coagulate' and go dry. In this state they could not form colonies when re-plated and were presumably dead.

4.2.3. Reaction of Salkowski reagent with IAA produced by Tn5 mutants of *Rhizobium*

The terms 'underproducer' and 'overproducer' are used in this thesis to denote a mutant strain of *R. phaseoli* 8002 which accumulated less or more IAA in the culture medium, relative to the wild-type strain, as determined by the results of Salkowski testing (**2.8.**)

Accumulation clearly is the balance between excretion of IAA into the medium and any re-assimilation and degradation of the excreted IAA that may occur.

Mutant colonies were tested for IAA production with Salkowski reagent seven days after diparental mating. Considerable colour variation, both lighter and darker than the wild-type, was found which suggested that the mutants contained levels of IAA different from the wild-type. Of the original 431 colonies which were tested with Salkowski reagent, 25 colonies gave a dark pink colour (putative overproducers) and 55 were colourless (putative underproducers). These colonies were re-plated and re-tested with Salkowski reagent, and the results are shown in **Table 4.3. Fig. 4.6.** shows the diversity of colour scored from (0) to (5) produced by the mutant colonies when tested with Salkowski reagent. Among the overproducers, twenty colonies gave scores of (4) and over. Four colonies gave a lower score of (3) and one colony, a score of (1). Of the underproducers, fifty-one of the colonies gave scores of lower than (3) with ten having a score of less than (1). For the wild-type, all colonies tested (20) consistently gave a score of (3).

Overproducers		Underproducers		Wild-Type	
Score	No. colonies	Score	No. colonies	Score	No. colonies
5	8	5	1	5	0
4	12	4	3	4	0
3	4	3	24	3	20
2	0	2	17	2	0
1	1	1	2	1	0
0	0	0	8	0	0
	Total = 25		Total = 55		Total = 20

Table 4.3. Scores from the second round of Salkowski testing of *R. phaseoli* 8002 Tn5 mutants. The scores of *R. phaseoli* 8002 wild-type and Tn5 mutants tested with Salkowski reagent (**2.8.2.**). A score of 5 denotes the brightest shade of pink while those with a score of 0 are colourless. **Fig. 4.6.** shows examples of colonies for each of the scores from (0) to (5).

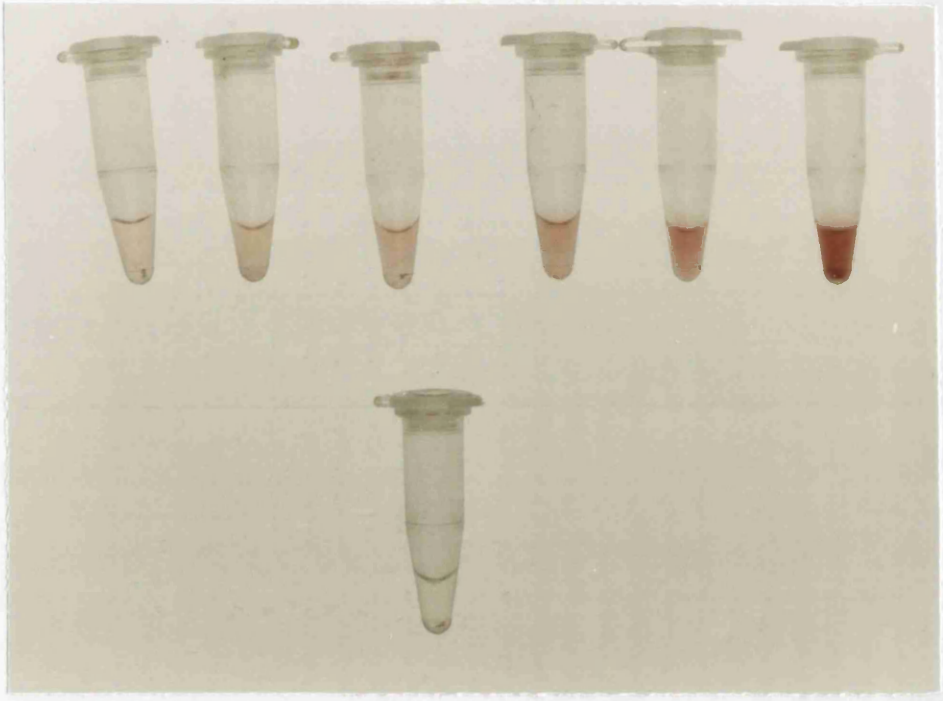


Fig. 4.6. Variation in colour intensity of mutant *R. phaseoli* 8002 tested with Salkowski reagent (2.8.2.) indicating levels of IAA different from the wild-type (designated score 3). Colour scoring for the tubes on the top row (left to right) were (1), (2), (2.5), (3), (4) and (5) while the colourless tube on the bottom row was designated (0).

The Salkowski method, as modified for use in the current research, gives only a comparative indication of IAA production. Consequently mutant colonies with scores of (0), (1) and (5) were selected for quantitative analysis of IAA production by high performance liquid chromatography (HPLC) combined with isotopic dilution (**2.5.**). The colonies were designated, A-I (putative overproducers) and J-T (putative underproducers) and grown on defined minimal media plates containing neomycin (30 µg ml^{-1}) before being transferred into neomycin (+) liquid medium.

4.3. Growth characteristics of mutants grown on solid medium

After re-testing with Salkowski reagent, the colonies of interest (A-T) were plated onto neomycin (30 µg ml^{-1}), necessary for the stability and maintenance of the transposon. **Table 4.4.** shows the variation in extracellular polysaccharide (EPS) production (scored + to ++++) by *R. phaseoli* 8002 Tn5 mutants on solid medium compared to wild-type production (+++). It was found that eight of the nine putative overproducers formed large, slimy colonies with high EPS production [(+++) or (++++)] with the exception of mutant F with a score of (++). All eleven of the putative underproducers gave 'dry' colonies, (+) or (++), indicative of reduced EPS production. Mutant H gave the most 'slimy' colonies while mutant J produced the 'driest' colonies.

Salkowski overproducers	EPS production	Salkowski underproducers	EPS production
A	+++	J	+
B	+++	K	+
C	+++	L	+
D	+++	M	++
E	++++	N	+
F	++	O	+
G	+++	P	+
H	++++	Q	+
I	++++	R	++
		S	+
		T	+

Wild-type EPS production (+++)

Table 4.4. The production of extracellular polysaccharide (EPS) on solid medium by *R. phaseoli* 8002 over and under IAA-producers (as judged by Salkowski testing). Assessment of EPS production by *R. phaseoli* 8002 mutants on solid medium plus tryptophan (200 mg l^{-1}) and neomycin (30 µg ml^{-1}). EPS production was judged visually and scored from (+) to (++++) (highest production) Wild-type 8002, cultured on neomycin (-) plates, was included for comparison.

4.4. IAA accumulation in the tryptophan-supplemented culture medium of *R. phaseoli* 8002 wild-type and Salkowski under and over IAA-producing mutants

4.4.1. IAA accumulation in the culture medium of *R. phaseoli* 8002 mutants A-T

R. phaseoli 8002 wild-type and Tn5 mutants (labelled A-T) were grown in tryptophan (+) shaking liquid culture (200 mg l^{-1}) (**2.2.**). All cultures were harvested during mid-log phase, at an optical density (500 nm) of approximately 1.5 units, regardless of the chronological age of the culture, as growth rates between mutants varied considerably. The majority of the IAA 'overproducers' (as judged by Salkowski testing in **Table 4.3.**)

were harvested approximately 24 h after flask inoculation. In contrast the underproducers, which grew more slowly, were harvested between 72 and 84 h (3-3.5 days) after inoculation. Wild-type 8002 was intermediate between these, reaching the required optical density approximately 36 h after inoculation. At harvest the number of cells per ml for all cultures varied from 6×10^8 to 1×10^9.

The accumulation of IAA per litre of culture medium and as a percentage of wild-type accumulation for each of the *Rhizobium* mutants is shown in **Table 4.5.** Of the nine putative overproducers, four (B, C, G and H) produced more IAA than the wild-type control, two (E and I) produced a similar amount and three (A, D and F) produced less.

The results for the putative underproducers were more consistent with those obtained during initial screening with Salkowski reagent, with significantly less IAA than the wild-type control accumulating in the culture medium of all Salkowski underproducers Production by nine of the eleven underproducers was less than 10% of the level of the wild-type and the remaining two (mutants J and P) were 17 and 20% respectively of the wild-type production.

The IAA accumulation, expressed as the quantity per 10^{11} cells, and as the percentage of wild-type accumulation is shown on **Table 4.6.** Of the nine putative overproducers, six (B, C, E, G, H and I) produced more than the wild-type and three (A, D and F) produced less. The underproducers all produced less than the wild-type. Nine (K, L, M, N, O, Q, R, S and T) produced less than 10% and the remaining two (J and P) produced 34 and 21% of the wild-type production respectively.

These results show that although the Salkowski test is a valuable and relatively quick technique for the initial screening of large numbers of colonies, it should be used in conjunction with an additional, quantitative technique. From these results, mutant H with

Mutant / wild-type	IAA µg l^{-1}	% of wild-type production*
Overproducers		
A	1,544	69
B	3,480	155
C	5,377	240
D	521	23
E	2,446	109
F	47	2
G	5,227	233
H	9,622	429
I	2,442	109
Underproducers		
J	390	17
K	114	5
L	23	1
M	24.5	1.1
N	28	1.2
O	28	1.2
P	451	20
Q	127	6
R	59	3
S	115	5
T	102	4.5
Wild-type		
8002 flask 1	2,163	100
8002 flask 2	2,324	100
Control	0	0

*2243.5 µg IAA per litre culture medium

Table 4.5. IAA accumulation (µg) per litre of tryptophan-supplemented culture medium of *R. phaseoli* 8002 wild-type and Tn5 mutants (A-T). The mean amount of IAA which accumulated (µg) per litre culture medium of *R. phaseoli* 8002 wild-type and Salkowski over and underproducing IAA mutants, A-T. Cells were shaken at 120 rpm at 27°C in defined liquid medium supplemented with tryptophan (200 mg l^{-1}) plus neomycin (30 µg ml^{-1}) (in the medium of the mutants). Uninoculated medium was extracted as a control. Variation between the values for replicate analyses of aliquots from the same sample was usually less than 5% and never more than 16%.

Mutant / wild-type	IAA µg per 10^{11} cells	% of wild-type production*
Overproducers		
A	140	62
B	680	300
C	867	382
D	77	34
E	948	418
F	7.7	3.4
G	628	277
H	1069	471
I	490	216
Underproducers		
J	78	34
K	11	4.9
L	1.7	0.8
M	2.5	1.1
N	2.4	1.1
O	2.9	1.3
P	47	21
Q	11	4.7
R	7.6	3.4
S	17	7.5
T	12	5.3
Wild-type		
8002 flask 1	228	100
8002 flask 2	226	100
Control	0	0

*227 µg IAA per 10^{11} wild-type cells

Table 4.6. IAA accumulation (µg) per 10^{11} cells of *R. phaseoli* 8002 wild-type and Tn5 mutants growing in tryptophan-supplemented medium. The mean amount of IAA (µg) which accumulated per 10^{11} cells of *R. phaseoli* 8002 wild-type and Salkowski over and underproducing IAA mutants, A-T. Cells were shaken at 120 rpm at 27°C in defined liquid medium supplemented with tryptophan (200 mg l^{-1}) plus neomycin (30 µg ml^{-1}) (in the medium of the mutants). Uninoculated medium was extracted as a control. Variation between the values for replicate analyses of aliquots from the same sample was usually less than 5% and never more than 16%.

429% (per litre) and 471% (per 10^{11} cells) and mutant L with 1% (per litre) and 0.8% (per 10^{11} cells) of wild-type IAA accumulation were chosen for further study.

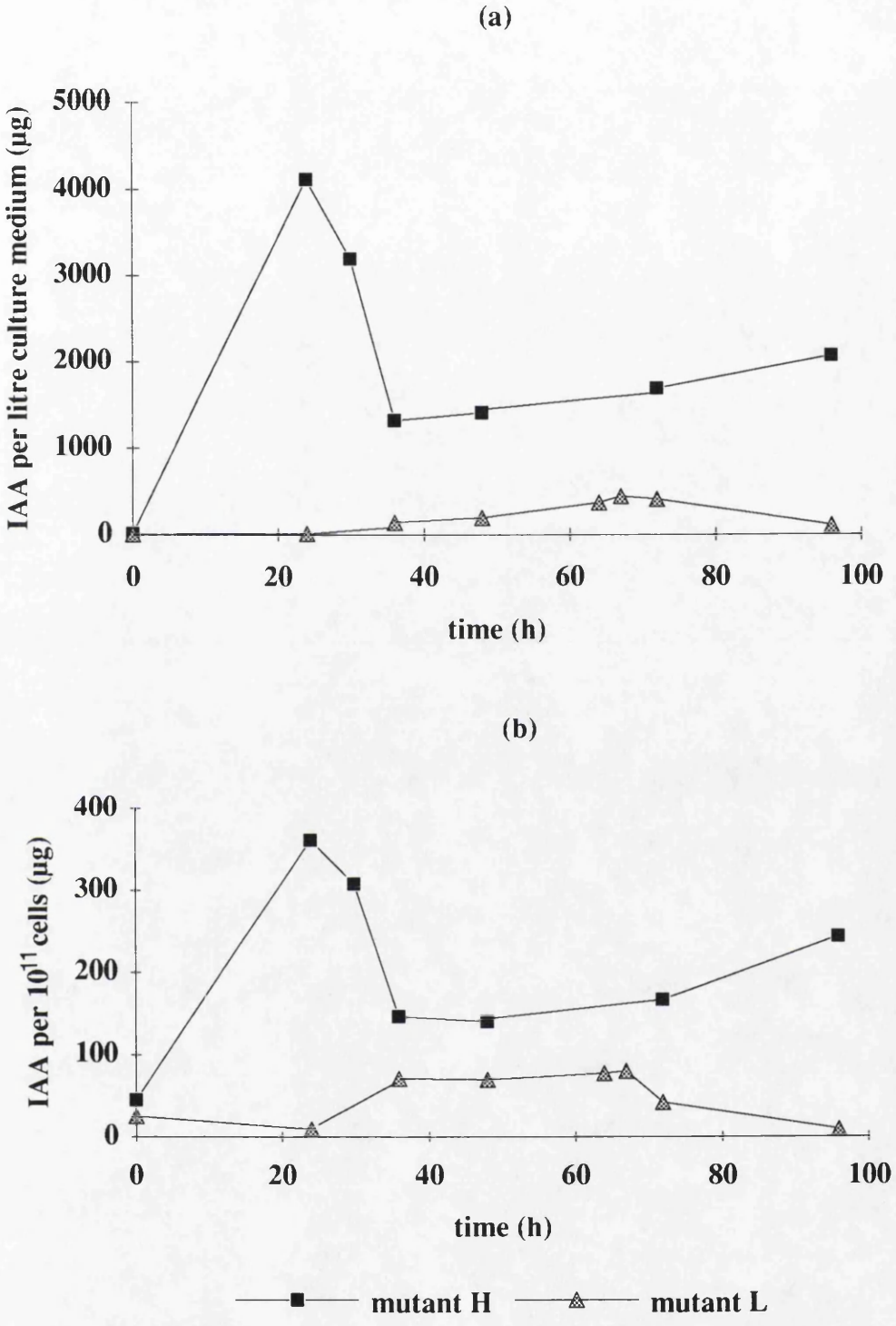
4.4.2. IAA accumulation in the culture medium of low (L) and high (H) IAA-producing mutant strains of *R. phaseoli* 8002 with time

IAA accumulation in the culture medium of low (mutant L) and high (mutant H) IAA-producing mutant strains of *R. phaseoli* (**4.4.1.**) over a time course was investigated. Cultures were grown in tryptophan plus (200 mg l^{-1}) shaking culture (120 rpm) (**2.2.3.**) and harvested and extracted at various intervals between 0 and 96 h after inoculation (**2.2.2.**), before quantitative analysis by RP-HPLC (**2.5.**).

The accumulation of IAA **(a)** per litre of culture filtrate and **(b)** to take account of differences in cell numbers between cultures, per 10^{11} cells of *R. phaseoli* 8002 high and low IAA-producing mutants at various intervals is shown on **Table 4.7.** and illustrated in **Figs. 4.7a.** and **4.7b.**

Time (h)	(a) mean µg IAA l^{-1}	(b) mean µg IAA per 10^{11} cells	range of sample values as a % of mean
mutant H			
0	**14**	**46**	**99-101**
24	**4,117**	**361**	**106-94**
30	**3,196**	**307**	**109-91**
36	**1,320**	**147**	**105-95**
48	**1,404**	**140**	**88-112**
72	**1,684**	**167**	**110-90**
96	**2,064**	**243**	**105-95**
mutant L			
0	**8**	**25**	**81-119**
24	**13**	**9**	**37-163**
36	**143**	**72**	**80-120**
48	**192**	**70**	**97-103**
64	**367**	**77**	**73-127**
67	**438**	**80**	**93-107**
72	**404**	**42**	**74-126**
96	**105**	**9**	**81-119**

Table 4.7. IAA accumulation (µg) (a) per litre of culture filtrate and (b) per 10^{11} cells of *R. phaseoli* 8002 high and low IAA-producing mutants with time. Cells were shaken at 120 rpm at 27°C in defined liquid medium supplemented with tryptophan (200 mg l^{-1}) plus neomycin (30 µg ml^{-1}) and cells harvested at various intervals between 0 and 96 h after inoculation.



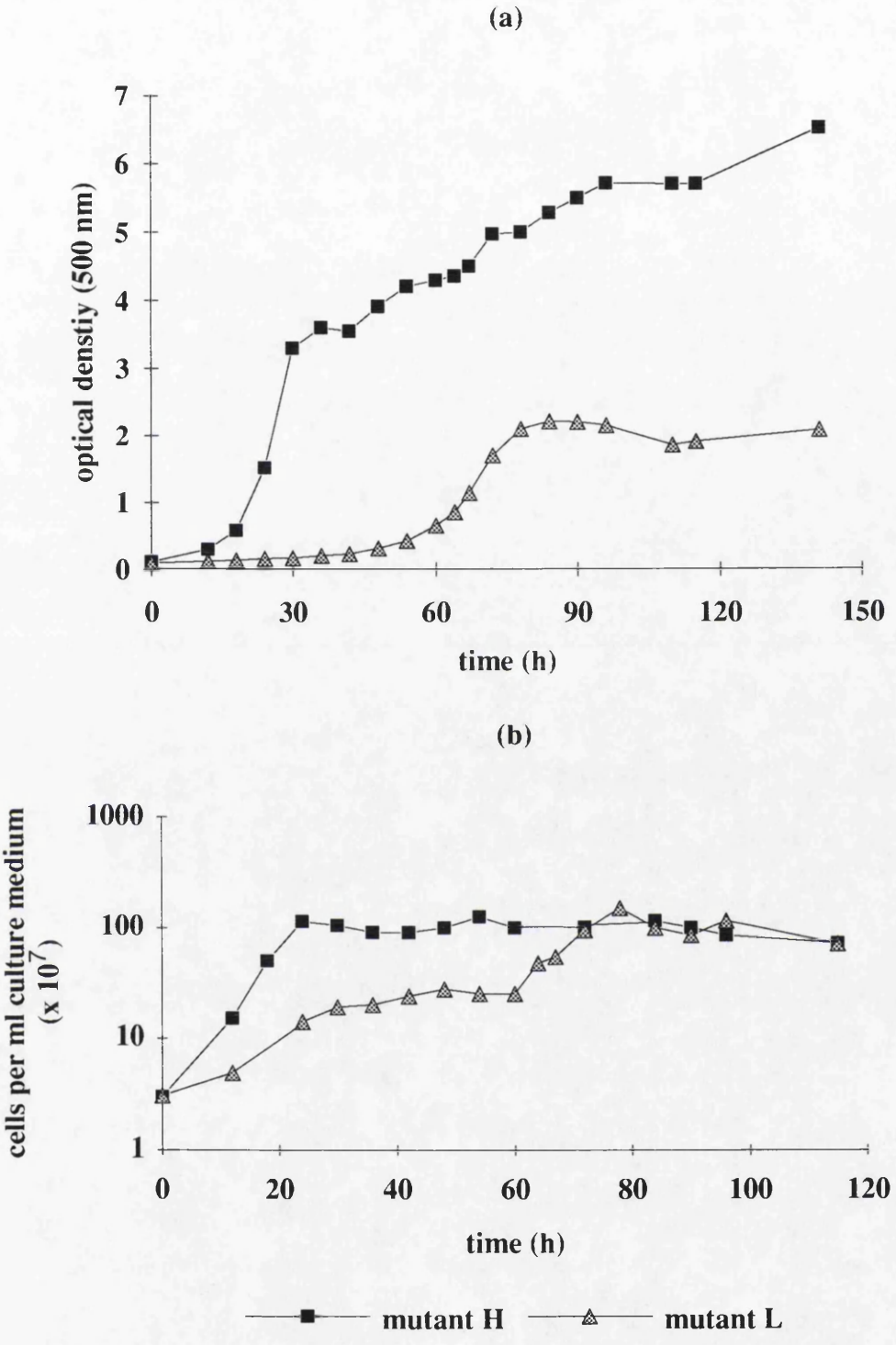
Figs. 4.7a. and 4.7b. IAA accumulation (µg) per litre of culture medium and per 10^{11} cells of high (H) and low (L) IAA-producing mutants of *R. phaseoli* harvested at intervals between 0 and 96 h after inoculation. Cells were shaken at 120 rpm and 27°C in defined liquid medium supplemented with tryptophan (200 mg/l) and neomycin (30 µg/ml).

The level of IAA in the culture medium of the high producer peaked 24 h after flask inoculation (4,117 µg per litre and 361 µg per 10^{11} cells) coinciding with the exponential phase of the culture between 15 and 30 h after inoculation (**Fig. 4.8.**). After this phase, the IAA level decreased sharply, reaching its lowest point at 48 h. The level of IAA then increased slightly to a level approximately half that accumulated (per litre) at 24 h.

For mutant L, maximum IAA accumulation occurred later than mutant H at 67 h (438 µg l^{-1} and 80 µg per 10^{11} cells), thus coinciding as before with the exponential phase of the culture (54 to 78 h) (**Fig. 4.8.**). After this, the level of IAA accumulation declined as the culture entered stationary phase.

A comparison of IAA accumulation in the medium of low and high IAA-producing mutants showed that at the point of maximum accumulation mutant H exhibited 9 times the amount of IAA present per litre and 4.5 times per 10^{11} cells compared with mutant L. The difference in IAA accumulation between the mutants was not as large as that predicted from earlier quantification (**4.4.1.**) where the amount of IAA harvested during exponential phase from the culture filtrate was 9622 µg l^{-1} and 23 µg l^{-1} for mutant H and L respectively. However, it is clear that there are significant differences in IAA accumulation between mutant L and H at all stages of culture growth (**Fig. 4.7.**).

Growth of the mutant cultures in liquid medium, as measured by the increase in optical density (500 nm) (**Fig. 4.8a.**) and viable cell counts (**Fig. 4.8b.**), were compared. From **Fig. 4.8a.**, it is clear that the mutant H culture growth rate is greater than mutant L with the exponential phase occurring between 15 and 30 h after inoculation, while mutant L has a less steep exponential phase which is 24 h in duration, between 54 and 78 h. In mutant H, as in the parent strain 8002, the optical density increases steadily and continues to rise during stationary phase. This increase continued until the final reading was taken at 141 h when the optical density had reached 6.52. Surprisingly, mutant L



Figs. 4.8a. and 4.8b. The optical density and mean cell number per ml of culture medium of *R. phaseoli* high (H) and low (L) IAA-producing mutants as a function of time (h). Cells were shaken at 120 rpm at 27°C in defined liquid medium supplemented with tryptophan (200 mg/l) and neomycin (30 µg/ml).

unlike strain 8002, did show a more typical sigmoid growth curve with the optical density levelling off at stationary phase. The optical density reading at 141 h post-inoculation was 2.08.

Growth curves of the number of cells per ml with time for mutants L and H are shown in **Fig. 4.8b.** For mutant H, the exponential phase began after a short lag phase and continued until 30 h, while mutant L had a long lag phase of 60 h, after which the exponential phase continued until 72 h after inoculation. Despite the large difference in the growth rate between the mutants, there was no difference in the number of cells per ml in the stationary phase of the culture (approx. 1 x 10^9 cells per ml).

4.5. Nodulation of *Phaseolus vulgaris* by *R. phaseoli* 8002 wild-type and high and low IAA-producing mutants.

4.5.1. Level of nodulation

Phaseolus vulgaris seedlings were inoculated with *R. phaseoli* 8002 wild-type, high (H) or low (L) IAA-producing mutants as described in **2.3.2.** As a control, ten uninoculated seedlings were grown with the inoculated plants under the same conditions, to check for accidental cross-infection between plants, for example during watering. Ten weeks after inoculation, the plant roots were examined for the presence of nodules. The roots of all plants inoculated with wild-type 8002 became infected, while only half of those inoculated with the mutant strains became infected. Examination of the roots revealed that the nodules produced following inoculation with the high and low IAA-producing mutants were morphologically similar to those produced by the wild-type (**Fig. 4.9.**).

The level of nodulation, mean approximate nodule diameter (mm) and the condition of plants nodulated by wild-type, mutant L and H are shown in **Table 4.8.** The level of nodulation was scored visually using a points system (0-4) with (0) representing no

Fig. 4.9. Nodulated roots of 10-week old *Phaseolus vulgaris* plants inoculated with *R. phaseoli* strain 8002 (A) wild-type, (B) mutant H, and (C) mutant L. Plants grown and inoculated as detailed in **2.3.1.** and **2.3.2.**

Rhizobium strain	nodulation score	mean nodule diam. (mm)	condition of plant and comment on nodulation
wild-type	4	4 - 6	plant healthy - large clusters of nodules
	4	3 - 5	plant healthy - even spread of nodules
	4	2 - 3	plant healthy - large clusters of nodules
	2	2	plant healthy - even spread of nodules
	1 - 2	2	plant yellowing - nodules on lower root only
	1	1	plant chlorotic - nodules on lower root only
mutant H	4	3 - 5	plant healthy - heavily nodulated
	3	3	plant healthy - even spread of nodules
	2	3	plant healthy - good nodulation
	0	0	plant chlorotic and unhealthy
	0	0	plant chlorotic and unhealthy
	0	0	plant dead
mutant L	4	3 - 4	plant healthy - large clusters of nodules
	3	3	plant healthy - nodules in clusters
	2	2 - 4	plant healthy - nodules evenly spaced
	0	0	plant chlorotic and unhealthy
	0	0	plant chlorotic and unhealthy
	0	0	plant chlorotic and unhealthy

Table 4.8. Nodulation of *Phaseolus vulgaris* by *R. phaseoli* 8002 wild-type, high (H) or low (L) IAA-producing mutant strains. The level of nodulation was scored using an arbitrary points system with (0) representing no nodulation and (4) representing the heaviest level of nodulation. Each strain was inoculated onto six plants and the condition of each replicate plant is recorded separately. The growth conditions of the plants are described in **2.3.1.**

nodulation and (4) indicating very heavy nodulation. None of the uninoculated control plants developed nodules and in addition, they were small and chlorotic. As reported earlier, all six of the plants inoculated with the wild-type strain developed nodules, although they appeared late on the peripheral roots of 2 plants. In contrast, only 3 out of 6 test plants were nodulated by each of the mutant strains, suggesting that these strains were less infective than the wild-type bacteria. In addition, plants nodulated by the wild-type strain had generally a better 'nodulation score' and the diameter of the nodules were generally larger than those inoculated by the mutants.

4.5.2. Nitrogenase activity

The nodulated roots of all plants tested (3 per strain) showed nitrogenase activity, as determined by acetylene reduction (**2.3.5.**) indicating that they were active in nitrogen fixation (**Table 4.9.**). Variability in the rates of reduction between plants inoculated with mutants H and L and **(1)** plants inoculated with wild-type and **(2)** each other, rendered the differences statistically insignificant ($p \leq 0.05$).

Strain	(a) C_2H_4 (nmoles) per plant per h (n=3)*	(b) C_2H_4 (nmoles) per g dry nodule wt. per h (n=3)*	(c) C_2H_4 (nmoles) per nodule per h (n=3)*
wild-type	7332 ± 2367	54148 ± 14962	19.1 ± 3.4
mutant L	5702 ± 1092	51233 ± 12172	15.8 ± 2.9
mutant H	4745 ± 2210	45803 ± 13383	19.2 ± 5.3

Table 4.9. Nitrogenase activity of roots nodulated by *R. phaseoli* 8002 wild-type, high (H) or low (L) IAA-producing mutant strains. The mean ± standard error of the amount of ethylene (C_2H_4) evolved (a) per plant per hour, (b) per gram dry nodule weight per hour and (c) per nodule per hour of *Phaseolus vulgaris* plants nodulated with *R. phaseoli* 8002 wild-type and high (H) and low (L) IAA producing mutant strains. Three plants were analysed per strain. Acetylene reduction assays and gas chromatography were carried out as described in **2.3.5.**

4.5.3. Plant biomass

Table 4.10. shows the dry weights, of shoot, root, nodule and total tissue and the number of nodules recorded for uninoculated and inoculated *Phaseolus vulgaris* plants. Two sets of figures are given for each of the mutant strains: **(1)** all plants were included in the analysis whether nodulated or not (n=6), **(2)** only nodulated plants were analysed (n=3, since only 50% of the plants inoculated with mutants L and H became infected).

1) There were no significant differences ($p \leq 0.05$) in the mean values for shoot, root and total plant weight **a)** between plants inoculated with the wild-type and those inoculated with the mutant strains and **b)** between those inoculated with mutants L and H. However, the dry weights of nodule tissue and the number of nodules of plants inoculated with the wild-type and those inoculated with mutant H were significantly different ($p \leq 0.05$). Nodule weight and nodule number of mutant H were 35 and 38% of wild-type. In contrast, although the average nodule weight and nodule number of mutant L were 44 and 69% of wild-type, the differences were not significant ($p \leq 0.05$).

2) When comparisons were made only with infected plants of mutant H and L (n=3), there were no significant differences ($p \leq 0.05$) for any of the criteria recorded **a)** between plants inoculated with the wild-type and those inoculated with the mutant strains and **b)** between those inoculated with mutants L and H.

Differences in growth between uninoculated controls in N (-) conditions, and inoculated plants would be expected once the seed reserves fall to a critical level. That this level had been passed in this experiment is shown by the significance ($p \leq 0.05$) of the differences for the mean values of most growth parameters between uninoculated plants and those inoculated plants which bore nodules. The only exception to this is the root weight which was not significantly different for plants in any treatment. As might be expected, when values for plants in the inoculated groups which remained un-nodulated were included in

	dry wt. g (mean ± standard error)				nodule number
inoculating strain	shoot wt.	root wt.	nodule. wt.	total wt.	(mean ± S.E.)
uninoculated (n=10)	0.668 ± 0.04 *a*	0.447 ± 0.04 *a*	0 *a*	1.121 ± 0.06 *a*	0 *a*
wild-type (n=6)	1.479 ± 0.22 *b*	0.538 ± 0.11 *a*	0.136 ± 0.02 *b*	2.153 ± 0.34 *b*	297 ± 58 *b*
mutant L (n=3)	1.622 ± 0.49 *b*	0.464 ± 0.19 *a*	0.121 ± 0.02 *bc*	2.207 ± 0.70 *b*	409 ± 151 *bc*
mutant L (n=6)	1.123 ± 0.31 *ab*	0.421 ± 0.09 *a*	0.060 ± 0.03 *bc*	1.604 ± 0.41 *ab*	205 ± 114 *bc*
mutant H (n=3)	1.403 ± 0.26 *b*	0.5 ± 0.12 *a*	0.096 ± 0.02 *bc*	1.999 ± 0.38 *b*	224 ± 42.5 *bc*
mutant H (n=6)	1.007 ± 0.21 *ab*	0.434 ± 0.06 *a*	0.048 ± 0.02 *c*	1.489 ± 0.29 *ab*	112 ± 54 *c*

Table 4.10. Dry weight of shoot, root, nodule, total plant weight and the number of nodules of *Phaseolus vulgaris* plants uninoculated and inoculated with *R. phaseoli* 8002 wild-type, high (H) or low (L) IAA-producing mutant strains. The dry weight (mean g ± standard error) of shoot, root and nodule and total plant dry weight and the number of nodules (mean ± standard error) of *Phaseolus vulgaris*. Two sets of figures are given for each of the mutant strains, n=6 where all plants grown were included in the statistics and n=3, where only the values for the nodulated plants were included. Within each column, those means which are followed by the same letter are not significantly different from each other ($p \leq 0.05$).

calculation of means, differences between some means were significant at $p \leq 0.05$, notably shoot and total weight of uninoculated plants and those inoculated with wild-type strain. The shoot weight of the uninoculated plants was 45% of the wild-type, while the total weight of the uninoculated plants was 52% of the wild-type.

4.6. Neomycin resistance in *R. phaseoli* 8002 wild-type and high and low IAA-producing mutant strains after re-isolation from the nodules of *Phaseolus vulgaris.*

The infecting bacteria were re-isolated from the nodules (**2.3.3.**). Each strain of bacteria was isolated from two separate plants and plated on defined minimal medium agar plates containing neomycin (Nm) (30 μg ml^{-1}) to determine the level of resistance in the bacterial population (**2.3.4.**). Identical aliquots of re-isolated mutant and wild-type cells were plated on Nm (-) plates as a control.

As shown in **Table 4.11.**, between 1.38 x 10^7 and 7.80 x 10^7 re-isolated bacteria (per ml of nodule suspension) grew on Nm (-) plates indicating that re-isolation had been successful. On Nm (+) plates, the level of Nm resistance in the mutant strains was low with less than 0.008% of the cells re-isolated from mutant H plants and less than 0.00007% of those from mutant L plants retaining Nm resistance. None of the re-isolated wild-type cells grew on the Nm (+) plates showing that there is no natural resistance in the population to Nm and that the plants inoculated with the wild-type were not cross-infected by resistant mutant bacteria.

Rhizobium strain	plant no.	cell no. ml^{-1} nodule suspension*		% of cells with Nm resistance
		Nm (+)	Nm (-)	
wild-type	1	0	1.86×10^7	0
	2	0	1.38×10^7	0
mutant H	1	4100	5.20×10^7	7.9×10^{-3}
	2	45	1.60×10^7	2.8×10^{-4}
mutant L	1	10	7.80×10^7	1.3×10^{-5}
	2	10	1.51×10^7	6.6×10^{-5}

*Nodules were crushed and the resulting suspension serially diluted and plated.

Table 4.11. Survival of re-isolated *R. phaseoli* strain 8002 wild-type and high and low IAA-producing mutant strains on Nm (+) and (-) plates. The survival of *R. phaseoli* strain 8002 wild-type and high (H) and low (L) IAA-producing mutant strains growing on Nm (+) (30 μg ml^{-1}) and (-) agar plates after re-isolation from the nodules of *Phaseolus vulgaris*.

The residual Nm resistance of the nodule bacterial population suggests that mutants L and H were responsible for infection of their host plants, as opposed to infection by the wild-type as a result of cross-contamination. As mentioned in **2.3.1.** and **2.3.2.**, strict precautions were taken to avoid cross-infection of strains by swabbing pots with ethanol, careful watering of plants and keeping plants inoculated with different strains separate in the growth-room. None of the uninoculated plants developed nodules and, as stated above, none of the cells re-isolated from the wild-type plants showed Nm resistance. It appears unlikely therefore that cross-contamination had occurred during the growth of the plants.

4.7. IAA accumulation in the culture medium of *R. phaseoli* 8002 re-isolated from nodules of *Phaseolus vulgaris* inoculated with wild-type and high and low IAA-producing mutant strains.

The accumulation of IAA in the culture medium of rhizobia re-isolated from the nodules of *Phaseolus vulgaris* inoculated with *R. phaseoli* 8002 wild-type and mutants L and H (**2.2.3.**) was investigated. As only a small proportion of the mutant cells retained Nm resistance (**Table 4.11.**), a mix of resistant and non-resistant colonies from Nm (-) plates were used to determine IAA production since it was thought that this would most nearly represent the situation 'in vivo'. Determination of IAA accumulation in the medium of Nm resistant cells was not undertaken since so few cells retained this property out of the bacterial population re-isolated from the nodules.

The data of **Table 4.12.** show that the amount of IAA accumulating in the culture medium of re-isolated cells of *R. phaseoli* 8002 wild-type and of the high and low IAA-producing mutants (H and L respectively) was much less than determined previously (**Tables 4.5. and 4.6.**). IAA accumulation in the culture medium of cells re-isolated from plants inoculated with the low IAA-producing mutant (L) was 50 and 27 times greater, per litre and per 10^{11} viable cells respectively, than the previous result. Conversely, IAA accumulation in the culture medium of cells re-isolated from plants inoculated with the high IAA-producing mutant (H), was only 20% (per litre) and 8% (per 10^{11} cells) of the previous result. However, the amount of IAA in the medium of re-isolated cells from plants inoculated with the wild-type also was only 68% (per litre) and 32% (per 10^{11} cells) of the level determined previously.

Although the mean amount of accumulated IAA in the medium of mutant L was apparently lower than the control, while that which accumulated in the medium of mutant H was apparently higher, overall, variability between cultures rendered differences in

IAA accumulation between (1) wild-type and mutant strains, and (2) between mutants, statistically insignificant ($p \leq 0.05$).

Wild-type / mutant strain	IAA	
	$\mu g\ l^{-1}$	μg per 10^{11} cells
wild-type flask 1	1,144	50
wild-type flask 2	1,912	93
mean ± S.E. (n=2)	1,528 ± 384	71.5 ± 21.5
mutant L flask 1	1,289	42
mutant L flask 2	1,038	50
mean ± S.E. (n=2)	1,163.5 ± 125	46 ± 4
mutant H flask 1	2,308	98
mutant H flask 2	1,615	73
mean ± S.E. (n=2)	1,961.1 ± 346	85.5 ± 12.5

Table 4.12. IAA accumulation in the culture medium of re-isolated *R. phaseoli* 8002 wild-type and high and low IAA-producing mutant strains. The amount of IAA which accumulated per litre and per 10^{11} cells in the culture medium of *R. phaseoli* 8002 wild-type and high (H) and low (L) IAA-producing mutant strains re-isolated from the nodules of *Phaseolus vulgaris*. Cells were grown in shaking, tryptophan supplemented liquid medium (200 mg l^{-1}) at 120 rpm and 27°C. Variation between the values for duplicate analyses from the same flask was between 2% and 6%.

4.8. Verification of transposition

4.8.1. Aims

The results shown in **Fig. 4.4a.** and **b** provide suggestive, but not conclusive evidence that transposition has occurred. If the ColE1 replicon was able to be maintained in *Rhizobium*, there would be less selection pressure on the transposon to integrate into the genome and it would be carried into the next generation by vegetative plasmid propagation rather than genomic replication. A further possibility is that is that part or all of pSUP2021 (**1.6.**) may have been integrated into the genome through recombination.

Verification that transposition had occurred and that the vector plasmid pSUP2021 had been lost is demonstrated in the following section.

The object of this section of work was to verify that Tn5 had transposed into the genome of mutants L and H, and was thereby responsible for the observed differences in IAA production between the mutants and the wild-type. In order to do this, genomic DNA was isolated from the mutants and the wild-type control, cut with restriction enzymes, Southern blotted onto a Hybond-N filter prior to hybridising with [^{32}P]-labelled probes (**2.9.**).

4.8.2. Digestion of *Rhizobium* DNA with restriction enzymes

Eight restriction enzymes were selected, because they were known, from the Tn5 restriction maps, to yield characteristic fragments that can be identified by hybridisation to a specific Tn5 probe. These enzymes were then used to determine which could cut *Rhizobium* DNA. **Fig. 4.10.** shows the resulting fragments, separated by a 1% agarose gel, from the digestion of *R. phaseoli* strain 8002 (wild-type) DNA by eight restriction enzymes. Uncut DNA (lane 2) remained near to the origin indicating that fragmentation had not occurred during isolation and that the DNA was therefore of good quality and high molecular weight. The following restriction enzymes, BamHI, ClaI, EcoRI, HindIII, PstI, SalI and SstI (lanes 3, 4, 5, 6, 8, 9, and 10 respectively) cut the *Rhizobium* DNA satisfactorily into ladders showing that there are many restriction sites for these enzymes. In contrast to this, there are apparently fewer restriction sites for KpnI (lane 7), as DNA digested with this enzyme did not ladder as well as with the other enzymes.

Once it had been established which enzymes could cut *Rhizobium* DNA, SalI and EcoRI were selected for the digestion of DNA from *R. phaseoli* strain 8002 wild-type, low (L) and high (H) IAA-producing mutants. The amount of 8002 DNA was increased from 1.4 to 5 µg for blotting. In addition to *Rhizobium* DNA, plasmid pSUP2021 (vector plasmid

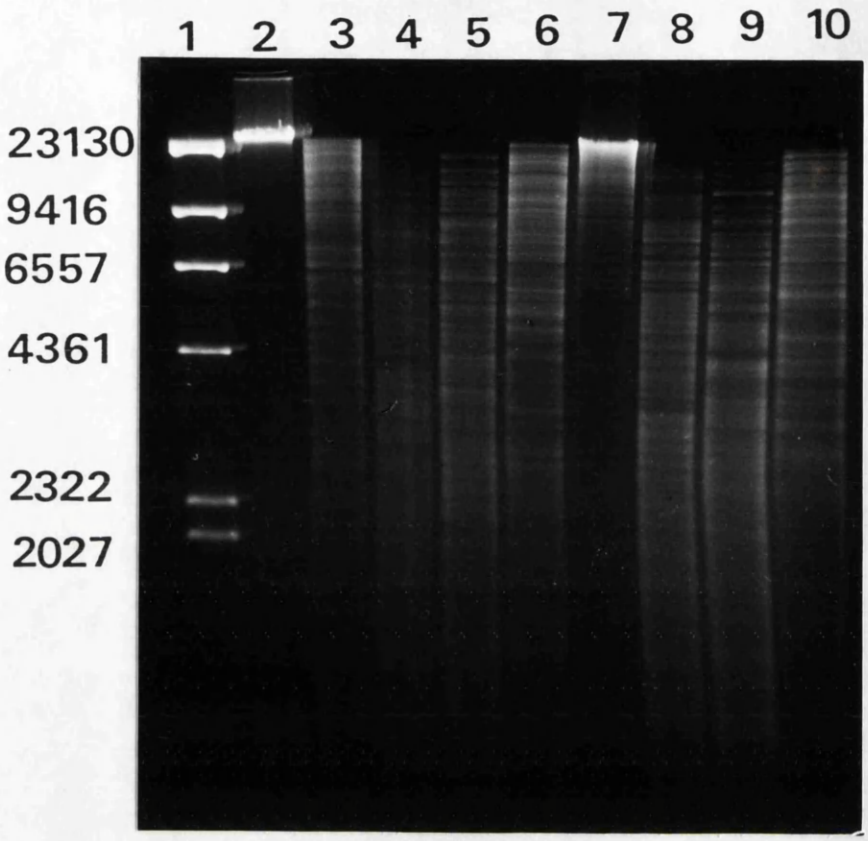


Fig. 4.10. Restriction digest of genomic DNA isolated from *R. phaseoli* strain 8002 wild-type. Fragments of lambda phage (λ cI857Sam7) DNA cut with HindIII were run in lane 1 to provide convenient size markers. The amount of DNA digested in lane 1 was 1 µg, while in the other lanes, this figure was 1.4 µg.

Lane 1 λ cI857Sam7 cut with HindIII

Lane 2 8002 uncut

Lane 3 8002 cut with BamHI

Lane 4 8002 cut with ClaI

Lane 5 8002 cut with EcoRI

Lane 6 8002 cut with HindIII

Lane 7 8002 cut with KpnI

Lane 8 8002 cut with PstI

Lane 9 8002 cut with SalI

Lane 10 8002 cut with SstI

plus transposon) and pSUP202 (vector plasmid minus transposon) were cut with BamHI and EcoRI. **Fig. 4.11.** shows the restriction digest of rhizobial and plasmid DNA separated by agarose gel electrophoresis. The DNAs from *Rhizobium* formed ladders on the gel while plasmids pSUP2021 and pSUP202 formed three and two bands respectively.

4.8.3. Hybridisation of *Rhizobium* DNA to probe pKan2

After separation, the DNA fragments were Southern blotted onto a filter prior to hybridisation with the labelled probes (pKan2 and pSUP202). To avoid having to continually refer to the 3.5 kb HindIII fragment of Tn5 (**Fig. 1.11.**) which is the central portion of Tn5 cloned into pKan2, the name pKan2 will be used to refer only to this probe for Tn5 and not to the whole plasmid. Probes were prepared (**2.9.6.**) and the hybridisation procedure carried out as detailed in **2.9.7.** and **2.9.8.**

Probing with pKan2, which is 100% homologous to Tn5 DNA, and subsequent autoradiography (**Fig. 4.12.**) showed that as expected there was no hybridisation to wild-type DNA, as it did not contain Tn5. The mutant DNAs (L and H) however hybridised to the probe and gave a signal. EcoRI does not cut within the 5.7 kb of the entire transposon and therefore only a single band is expected, SalI however cleaves the transposon at position 2684, i.e. approximately mid-way, and therefore yields 2 bands on hybridisation. Where mutant DNA was digested with EcoRI (lanes 4 and 6) there was a single hybridisation band and where it had been digested by SalI (lanes 5 and 7) there were two bands. The Tn5 has inserted into 7.3 and 8.9 kb EcoRI fragments of the genomes of mutant H (lane 4) and mutant L (lane 6) respectively. Similarly digestion with SalI revealed that the transposon was inserted into a 2.9 and 8.6 SalI fragment of mutant H (lane 5) and mutant L (lane 7) respectively. Plasmid pSUP2021 (+Tn5), was cut with EcoRI and BamHI. Two hybridisation bands are expected as EcoRI cuts on either side of the Tn5 and BamHI cuts the Tn5 between these points at position 3070.

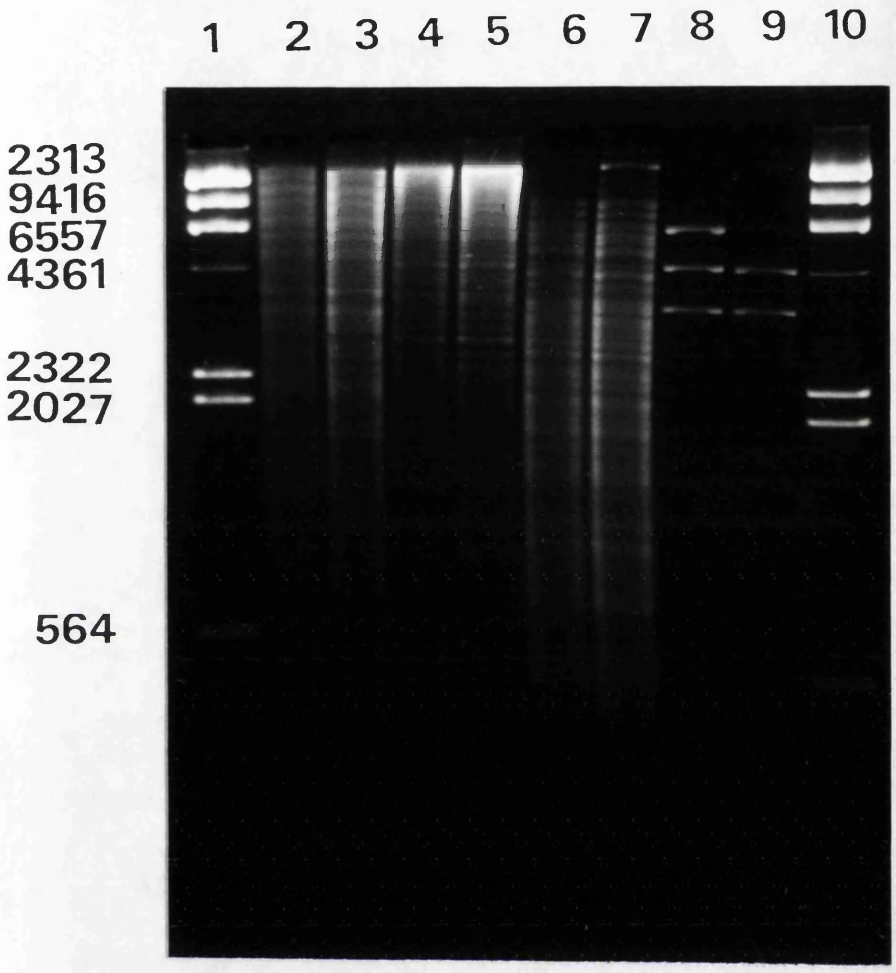


Fig. 4.11 Restriction digest of genomic DNA isolated from wild-type and mutant strains L and H of *R. phaseoli* strain 8002. Fragments of lambda phage (λ cI857Sam7) DNA, cut with HindIII, were run in lane 1 to provide convenient size markers. The amount of DNA digested was 1 µg (lanes 1 and 10), 0.1 µg (lanes 8 and 9) and 5 µg (lanes 2-7).

Lane 1 λ cI857Sam7 cut with HindIII

Lane 2 Wild-type cut with EcoRI

Lane 3 Wild-type cut with SalI

Lane 4 Mutant H cut with EcoRI

Lane 5 Mutant H cut with SalI

Lane 6 Mutant L cut with EcoRI

Lane 7 Mutant L cut with SalI

Lane 8 pSUP2021 cut with BamHI and EcoRI

Lane 9 pSUP202 cut with BamHI and EcoRI

Lane 10 λ cI857Sam7 cut with HindIII

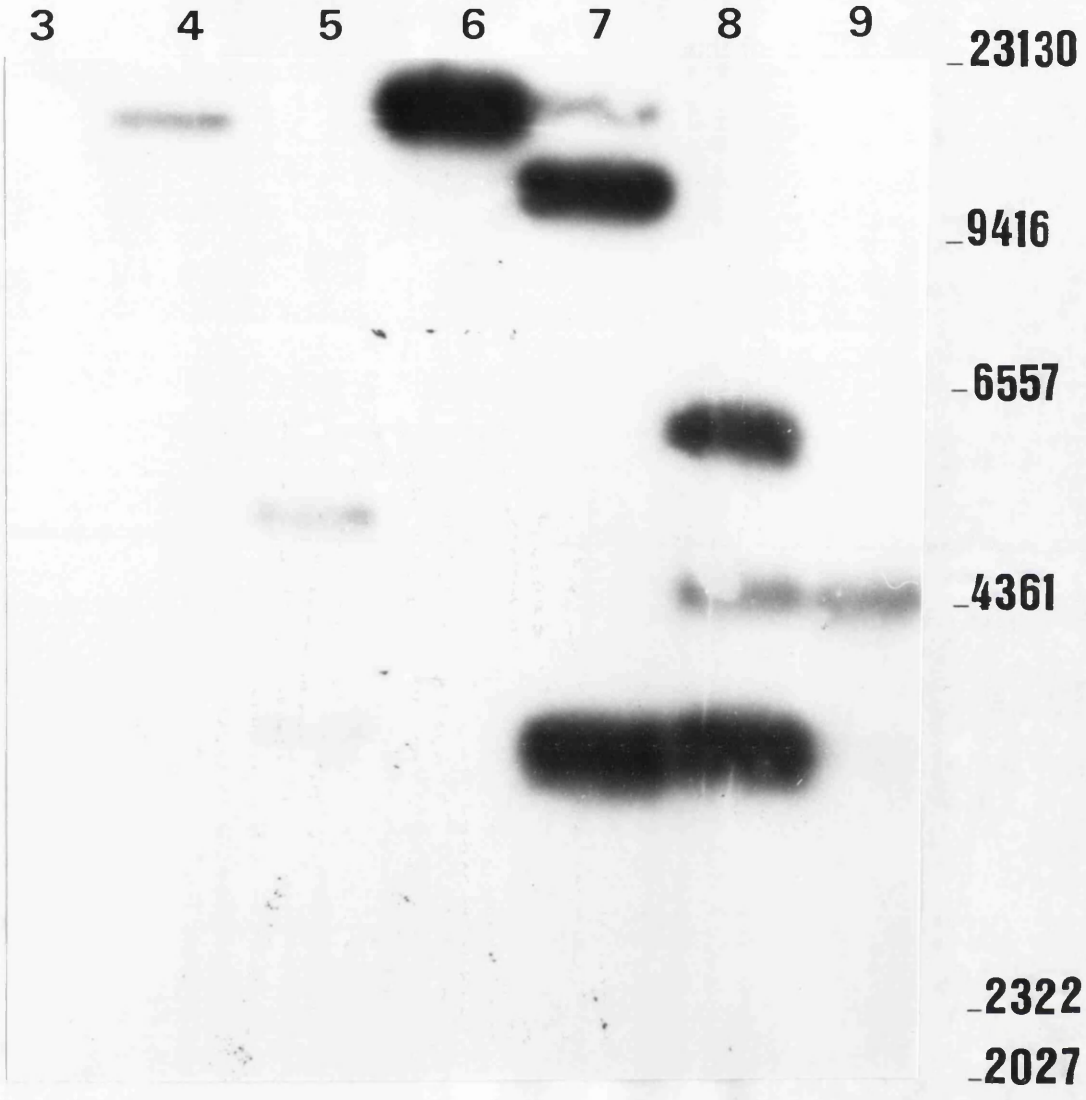


Fig. 4.12. Autoradiograph of the filter probed with [^{32}P]-radiolabelled pKan2. The filter was Southern blotted from the gel shown in **Figure 4.11.** A signal was obtained from the DNA of both high and low IAA-producing mutant strains showing that Tn5 was present in the genomic DNA. No signal was obtained from the wild-type showing that Tn5 was absent. A full explanation of the banding pattern is given in the text (**4.8.3.**)

However, pSUP2021 had three hybridisation bands, two that gave bright signals and one which gave a faint signal. As only 2 bands were expected to hybridise to pSUP2021, there must be a small degree of homology between probe pKan2 and a band found outwith the transposon, i.e. part of the vector, to account for the faint signal obtained. There was no hybridisation signal from pSUP202, except for the faint band mentioned above, indicating that Tn5 was not present on this plasmid. These results confirmed that Tn5 was present in mutants L and H and plasmid pSUP2021 and absent from the DNA of the wild-type and pSUP202.

4.8.4. Hybridisation of *Rhizobium* DNA to probe pSUP202

After probe pKan2 had been removed, i.e. denatured by washing in dilute NaOH solution (**2.9.9.**), the filter was autoradiographed to ensure that the probe had been completely removed. The filter was then re-hybridised to probe pSUP202 which hybridises to plasmid DNA. As expected, signals were obtained only from pSUP2021 (lane 8) and (lane 9) pSUP202 DNA. As no hybridisation signal was obtained from the genomic DNA, the vector plasmid had been lost from the rhizobia, and transposon DNA only was present in the genome of mutants L and H. Thus Tn5 had been replicated on the genome and not through vegetative replication of the plasmid.

4.9. Conclusions

The work reported in this chapter describes the isolation of *Rhizobium phaseoli* high and low IAA-producing mutants created by transposon mutagenesis. Initial screening of the mutants with Salkowski reagent and subsequent quantitative analysis by HPLC, revealed significant differences in IAA accumulation between wild-type and mutant bacteria. Although unfortunately a completely IAA-minus mutant was not isolated, a number of colonies with altered levels of IAA accumulation, both higher and lower than the wild-

type, were found. In these mutants there was a strong correlation between IAA accumulation and EPS secretion.

Hybridisation analysis of the highest and lowest mutants, H and L respectively, verified that these mutations were the result of transposon insertion into the genome of *R. phaseoli* with concomitant deletion of the vector plasmid.

Analysis of IAA accumulation with time in the highest and the lowest IAA-producer (as determined by HPLC) revealed that although the differences in IAA production between mutants were not as large as those predicted from previous results, they were nevertheless significant at all stages of culture growth. The differences in IAA accumulation were per litre and also per 10^{11} cells and therefore were not due simply to differences in culture growth rate.

Mutants L, H and wild-type bacteria were inoculated onto *Phaseolus vulgaris* in order to determine whether there is a correlation between IAA production and nodulation ability by the bacterium. No significant differences were found in the level of nitrogen fixation or dry weight between plants inoculated with wild-type and mutant bacteria. The wild-type however, was apparently more infective than the mutant strains (100% nodulation by wild-type and 50% by each of the mutant strains). Re-isolated bacteria from these nodules revealed that only a residual resistance to neomycin remained in the population. In addition, analysis of IAA accumulation in the culture medium of re-isolated bacteria (a resistant and non-resistant mixture of mutants representing the situation *in vivo*), revealed no significant differences between re-isolated strains.

CHAPTER 5

DISCUSSION

5.1. Indole accumulation in the culture medium of *Rhizobium phaseoli* in tryptophan (+) and (-) medium

5.1.1. Identification of the indoles produced

Co-chromatography with known indolic standards during reversed and normal phase HPLC analysis led to a tentative identification of the compounds produced by *Rhizobium phaseoli* in tryptophan (+) and (-) medium (**Fig. 3.3.**). Subsequent MS analysis of these compounds revealed that the molecular weight and fragmentation pattern corresponded to those of authentic indole acetic acid (IAA), indole lactic acid (ILA), indole ethanol (IEt) and indole aldehyde (IAld) and thus conclusive identifications were made (**Table 5.1.**). In addition, evidence was found for the possible presence of indole methanol (IM). This spectrum of indoles was found in both the tryptophan (+) and (-) medium, although production was greatly stimulated in tryptophan (+) medium (**3.4.**).

Indolic compound	(a) Present study	(b) Ernstsen *et al.*, 1987	(c) Badenoch-Jones *et al.*, 1982
IAA	+	+	+
IEt	+	+	+
ILA	+	ND	+
IAld	+	+	+
IM	+	+	ND
ICA	ND	ND	+
IGA	ND	ND	+
IGoxA	ND	ND	+
IPyA	ND	ND	+
IAAm	ND	ND	ND

Table 5.1. Indolic compounds detected in the culture medium of *Rhizobium phaseoli* 8002 (a) in both (+) and (-) tryptophan medium, (b) in (-) tryptophan medium, and *R. trifolii* and *R. leguminosarum* (c) in (+) tryptophan medium.

The spectrum of indoles detected in (+) and (-) medium of the present study in *R. phaseoli* 8002 was identical to those identified by Ernstsen *et al.* (1987) in tryptophan (-) culture medium (**Table 5.1.b.**), with the exception of ILA, and thus this is this first identification of this compound in this species [in both (+) and (-) tryptophan medium], although it has been detected previously in *R. trifolii* and *R. leguminosarum* (**Table 5.1.c.**) (Badenoch-Jones *et al.*, 1982a) and in the actinomycete *Frankia* sp. HFPArI3 (Berry *et al.*, 1989).

There was less similarity however, in the indole profile between the current study and those found in the culture medium of *R. trifolii* and *R. leguminosarum* (**Table 5.1.c.**) (Badenoch-Jones *et al.*, 1982a), mainly because fewer indoles were detected in the present work. Of the 8 indoles detected in *R. trifolii* and *leguminosarum*, only 4 were found in the present study. In addition, although IM was detected in the current work, it was not detected by Badenoch-Jones and her co-workers (1982a). These differences could be due to variations between species or to differences in culture conditions (Ernstsen *et al.*, 1987).

5.1.2. Quantitative determination of production

Tryptophan was shown to have a stimulatory effect on the level of indolic compounds secreted into the medium by the bacterium. Comparison of indole accumulation in tryptophan (+) and (-) medium revealed that relative accumulation was not identical for each indole. In tryptophan (+) medium the increase in ILA accumulation was larger than the increase in IAA and IEt accumulation which was in turn greater than the accumulation of IM. Possible reasons for these differences are discussed in **5.4.**

Badenoch-Jones *et al.* (1982b) detected between 162 and 711 $\mu g\ l^{-1}$ IAA in the culture medium of wild-type *R. trifolii* and *leguminosarum* strains growing in tryptophan (+) medium (56 $mg\ l^{-1}$). These figures are similar to those of Wang *et al.*, (1982) who

quantified IAA accumulation in wild-type strains of *R. leguminosarum* at between 104 - 849 μg l^{-1} in tryptophan (+) (200 mg l^{-1}) medium and at 80 - 550 ng l^{-1} in tryptophan (-) medium. These figures are lower than those of this project where 1.5 - 2 mg l^{-1} and 20 - 21 μg l^{-1} IAA were recorded in tryptophan (+) medium (200 mg l^{-1}) and tryptophan (-) medium respectively. As stated previously, these differences may be due to differences between biovars of *R. leguminosarum,* or as in the Badenoch-Jones study (1982b) to different levels of tryptophan supplementation in the culture medium.

5.1.3. Possible modifications of analytical techniques

MS analysis of the samples revealed the contamination of both the IAA and the IEt samples by IAld (**Figs. 3.4.1.** and **3.4.2.**). As IAld does not fluoresce (Burnett and Audus, 1964), it was not detected prior to MS analysis. The retention times of IEt, IAld and IAA are relatively close (**Fig. 3.2.**) and hence it is probable that IAld, undetected by the fluorescence monitor, was collected along with the IAA and IEt. However, this does not explain why IAld was found in both the acidic and neutral/basic fractions. The partitioning of IAld into both these fractions during solvent extraction (**Fig. 2.1.**) suggests that the IAld is not removed efficiently from the medium into the neutral/basic fraction and is therefore carried over into the acidic fraction. In future work involving indoles it would be advisable to ascertain the effectiveness of the partitioning procedure for each indole, and in addition, to monitor the presence of indoles with both U.V. and fluorescence detectors.

5.1.4. Conclusions from studies of indole production

It is clear that the addition of tryptophan to the culture medium leads to a stimulation of IAA production. However, even in the absence of both exogenous tryptophan and host plant, the bacteria still synthesise IAA, channelling tryptophan away from protein synthesis to act as the precursor. This apparently wasteful synthesis suggests that either IAA *per se,* or a biosynthetic or catabolic intermediate, may be required by the bacterium

itself. Alternatively IAA production might be a mechanism for the removal of excess tryptophan from the cell.

5.2. The effect of gibberellic acid (GA_3) on IAA production by *R. phaseoli* 8002

It was postulated that the addition of GA_3 to the culture medium of *Rhizobium* would lead to increased IAA production. Law and Hamilton (1984) found that the application of GA_3 to dwarf pea increased IAA levels 8-fold. They postulated that the addition of GA_3 caused the racemisation of L-tryptophan to D-tryptophan which they hypothesised was the immediate precursor of IAA (Law and Hamilton, 1985)(**Fig. 1.13.**). The results of the current study (**Table. 3.8.**) provide little evidence that synergism between IAA and gibberellin occurs in *R. phaseoli in vitro*. There was a great deal of variation between flasks of the same treatment so that although the first experiment pointed to a slight stimulation in production when treated with 10^{-6} M GA_3, this result was not obtained in the subsequent experiment. Indeed, it appeared that higher concentrations of gibberellic acid had slightly inhibitory effects on production. It is possible that synergism between IAA and GA_3 would occur if concentrations of GA_3 other than those tried previously were investigated. Additionally, these results do not rule out the possibility of synergism between GA_3 and IAA in the nodule as both of these compounds are known to be synthesised in this tissue (Atzorn *et al.*, 1988).

5.3. Metabolism of tryptophan and IAA by *R. phaseoli in vitro*

5.3.1. [^{3}H]-tryptophan feed to *R. phaseoli*

The addition of labelled tryptophan to cultures of *R. phaseoli* resulted in the radiolabelling of IAA, ILA, IAld, IEt and IM. These labelled products were identical to

the spectrum of indoles detected in the culture medium of the bacterium (**Table 5.1.**). The majority of the radioactivity was incorporated into the major metabolites, IAA and ILA.

Ernstsen *et al.* (1987) previously reported the conversion by *R. phaseoli* of labelled tryptophan to IEt, IAA and IM and labelled IEt to IAA and IM, however, the conversion of labelled tryptophan to ILA and IAld, as detected in this project, has not been reported before.

5.3.2. [^{14}C]-IAA feed to *R. phaseoli*

The addition of radiolabelled IAA to *Rhizobium* cultures resulted in its conversion to IEt, IAld and IM. The conversion of IAA to IM is in agreement with Ernstsen *et al.* (1987) who found that labelled IAA was converted to IM. However, the conversion of IAA to IAld and IEt as reported in the current work, have not been demonstrated before, although IEt and IAld have previously been detected in the culture medium of *R. phaseoli* (Ernstsen *et al.*, 1987) and in the present work (**Table 5.1.**). This result is in agreement with **5.3.1.**

5.4. Proposed pathways of IAA metabolism in *R. phaseoli* 8002

The expected intermediates suggested for each of the IAA **(a)** biosynthetic and **(b)** degradative pathways have been described in detail previously (**1.1.**).

5.4.1. IAA biosynthesis

Identification of the major indoles which accumulate in the culture medium of *R. phaseoli* and the metabolic products of labelled tryptophan and IAA feeds suggest that the IAA biosynthetic pathway operating in *R. phaseoli* 8002 *in vitro* from tryptophan is via IPyA and IAAld (**1.1.2a.**) (**Fig. 5.1.**).

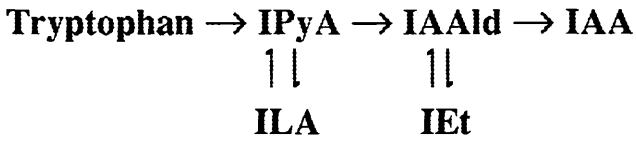


Fig. 5.1. Proposed pathway of IAA biosynthesis in *Rhizobium phaseoli* 8002.

Neither indole pyruvic acid (IPyA) nor indole acetaldehyde (IAAld) were detected, however these compounds are known to be highly unstable and break down easily during sample purification (Kaper and Veldstra, 1958; Dullaart, 1967). IPyA has been detected in *R. trifolii* and *leguminosarum* medium by GC/MS analysis (Badenoch-Jones *et al.*, 1982a) and in *R. meliloti* by TLC (Williams and Signer, 1990), although Ernstsen *et al.* (1987) did not detect this compound in *R. phaseoli*. Although, IAAld was detected by Williams and Signer (1990), both Badenoch-Jones *et al.* (1982a) and Ernstsen *et al.* (1987) were unable to detect IAAld in the culture medium of all *Rhizobium* species tested. As reported in **5.1.1.**, ILA and IEt, the corresponding reduced compounds of IPyA and IAAld respectively (Bandurski and Nonhebel, 1984), were detected in the current study suggesting that they are metabolic products of the reduction of IPyA and IAAld.

Tryptamine, which is the alternative intermediate between tryptophan and IAAld (**Fig. 1.3.**), was not detected as either an endogenous compound or as a metabolite of tryptophan. This observation, coupled with the detection of ILA in the culture medium and the conversion of labelled tryptophan to ILA (the reduced form of IPyA) suggests that the pathway of IAA synthesis is via IPyA rather than tryptamine (**Fig. 1.3.**). In addition, tryptamine was not detected in either of the two recent studies of rhizobial production and indole metabolism (Badenoch-Jones *et al.*, 1982a; Ernstsen *et al.*, 1987).

If the IPyA-IAAld pathway of IAA biosynthesis is operating in *R. phaseoli* strain 8002, this is clearly different from the pathway operating in a number of other bacteria where IAA is synthesised from tryptophan with IAAm as the primary intermediate: *Pseudomonas savastanoi* (Morris, 1986), *Agrobacterium tumefaciens* (Morris, 1986) and *Bradyrhizobium japonicum* (Sekine *et al.*, 1988). Despite the stability of the molecule, IAAm was not detected as an endogenous constituent or as a tryptophan metabolite in the current study. This again is in agreement with previous studies where IAAm was not detected in the rhizobial culture medium (Badenoch-Jones *et al.*, 1982a; Ernstsen *et al.*, 1987), moreover, cultures did not convert labelled IAAm to IAA (Ernstsen *et al.*, 1987). Additionally, IAAm and IAAm hydrolase activity were detected in *Bradyrhizobium japonicum,* but were not detected in any of the *Rhizobium* species examined by Sekine *et al.* (1988). It seems therefore that the pathway operating in *Bradyrhizobium japonicum* is different from that in *Rhizobium* despite the close taxonomic relationship between them (Kreig and Holt, 1984). However, it has been postulated that genes for both pathways are present in both species. Firstly, it has been suggested that *Rhizobium* may also possess the gene for IAAm hydrolase (Sekine *et al.*, 1988) but which may only be expressed under certain culture conditions or is perhaps induced only in the presence of host plant metabolites (Sekine *et al.*, 1988). At present there is no evidence to support this hypothesis. Secondly, IPyA has been detected in *Bradyrhizobium japonicum* (Kaneshiro *et al.*, 1983) implying that the IPyA pathway of IAA biosynthesis may also operate in these bacteria.

The IPyA pathway, apparently operating in *R. phaseoli* 8002 in the current study, is the predominant route of IAA synthesis in higher plants (Schneider and Wightman, 1978; Sandberg *et al.*, 1987). In contrast, as discussed previously, the major pathway of synthesis in bacteria investigated thus far appears to be via IAAm. Evidence however of another bacterium with the IPyA biosynthetic pathway, was found recently with the discovery that *Frankia* produces a similar spectrum of indoles in culture to those found

in *R. phaseoli* (Berry *et al.*, 1989). This suggests that IAA synthesis in this micro-organism is, like *R. phaseoli*, via the IPyA-IAAld pathway.

5.4.2. Possible roles of IEt and ILA

a) IEt

What benefit could the production of IEt have for *Rhizobium*? It has been suggested that the reversible reduction of IAAld to IEt is an IAA regulatory device (Rayle and Purves, 1968). This hypothesis assumes that IEt plays the role of an auxin storage compound which under certain conditions may be oxidised to IAAld and IAA. The production of IEt has certain advantages as a means of regulating IAA biosynthesis. IEt is a chemically stable molecule and thus has an advantage over IPyA or IAAld as a storage product (Rayle and Purves, 1968). In addition, it is non-toxic at high concentration and is not attacked by peroxidase. Hence, IEt may accumulate and be available for rapid conversion to IAA as required. Furthermore, IEt might participate in the regulation of IAA biosynthesis as IEt production represents a branch point and may be subject to allosteric control by the final product of the pathway. It may be postulated that IAA acts as an allosteric inhibitor of IAAld dehydrogenase, the enzyme catalysing the conversion of IAAld to IAA. As the IAA level increases, the dehydrogenase would be inhibited, causing the preferential formation and storage of IEt (Rayle and Purves, 1968).

Enzymic reduction of IAAld to IEt has been described (Brown and Purves, 1980) and in addition, the enzymic oxidation of IEt to IAAld has been detected in higher plants (Percival *et al.*, 1973) and the fungus *Phycomyces blakesleeanus* by Schramm *et al.* (1987) who detected an IEt oxidase subject to feed-back regulation by IAA and suggested that IEt was thus a 'storage pool' for IAA in *P. blakesleeanus*. The conversion of IEt to IAA has been detected in *R. phaseoli* (Ernstsen *et al.*, 1987) and in addition IEt

has been shown in the current study to be excreted in relatively high quantities into the medium (**Table 3.5.**) and hence IEt might be an important regulatory compound in this species.

b) ILA

The large increase in ILA production in the presence of relatively high amounts of tryptophan (200 mg l^{-1}) (**Table 3.5.**) implies that, as has been suggested for plants (Schneider and Wightman, 1978), the conversion of tryptophan to ILA in *Rhizobium* is a 'side shunt' away from IAA production (**Fig. 5.1.**). It is possible that the bacterium regulates or limits IAA production by converting excess tryptophan to ILA as it is known that the step between IPyA and ILA is reversible (Bandurski and Nonhebel, 1984). Tryptophan levels in the medium are higher than the levels of IAA, and one might predict that the principal point of regulation of auxin synthesis would be found in the conversion of tryptophan to IPyA. Furthermore, ILA may be a compound active in the physiology of the bacterium or in symbiosis.

Wightman (1964) proposed that in tomato, IEt is formed through the decarboxylation of ILA and hence may be an intermediate in IAA formation rather than a side branch and thus it is possible that the detection of ILA and IEt (and conversely the non-detection of IPyA and IAAld) are indications that this pathway is operating in this species. However, this pathway has not been detected in any other species and hence remains speculative.

5.4.3. IAA catabolism

The detection of IAld and IM in the culture medium of *R. phaseoli* and the metabolic conversion of labelled IAA to IAld and IM suggests that IAA is converted sequentially to IM, IAld, and ICA in *R. phaseoli* 8002 *in vitro* (**1.1.3a.**) (**Fig. 5.2.**). IAA catabolism appears therefore to be primarily decarboxylative involving loss of the acidic side chain carboxyl group.

IAA → IM → IAld → ICA

Fig. 5.2. Possible pathway of IAA catabolism by *Rhizobium phaseoli* 8002.

The conversion of IAA to IM has been demonstrated previously in this species (Ernstsen *et al.*, 1987), and the conversion of IAA to IAld is reported in this thesis (**5.3.2.**). However, the final step from IAld to ICA remains speculative as ICA has not been identified as an IAA metabolite or as an indolic component from the culture medium of *R. phaseoli* 8002 in either the current study or in the work of Ernstsen *et al.* (1987), although ICA has been detected from the culture medium of *R. trifolii* and *R. leguminosarum* (Badenoch-Jones *et al.*, 1982) (**Fig. 5.1.**).

In addition to radiolabelled IAld and IM, radiolabelled IEt was also detected as a product of rhizobial IAA metabolism (**Fig. 3.6.3.**). IEt was not detected as product of IAA metabolism by Ernstsen *et al.* (1987) in *R. phaseoli*. The conversion of IEt to IAAld is reversible (**Fig. 5.1.**)(Sandberg *et al.*, 1987), in addition the results presented in this thesis suggest that the step between IAAld and IAA is also reversible. The possibility exists however, that IAA has been degraded non-enzymatically to IAAld as it is known that IAA can break down into a number of indolic products (Kaper and Veldstra, 1958); this IAAld is then converted to IEt.

5.4.4. Alternative pathways of biosynthesis in tryptophan-supplemented medium

It was postulated that tryptophan enrichment of the culture medium might lead to the expression of alternative pathways of IAA synthesis. However, the results reported in this thesis, provide no evidence in support of the existence of other pathways in tryptophan-supplemented *R. phaseoli* culture medium, at least in this strain of *Rhizobium* growing under these conditions. There was in addition, no evidence from *Rhizobium* to

support the theory of two separate pathways of IAA biosynthesis, as has been suggested for *Bradyrhizobium japonicum* (Sekine *et al.*, 1988).

5.4.5. Further experimentation

If time allowed, a labelled pulse-chase experiment would have been carried out to follow the conversion of tryptophan to the indolic compounds. This would allow the sequence of intermediates to be followed closely.

Additionally, *Rhizobium* DNA could be screened with auxin gene probes isolated from other species, for example, *Pseudomonas savastanoi* IAA biosynthetic genes *iaaM* and *iaaH* (**Fig. 1.9.**). If there was sufficient homology between the probe and the *Rhizobium* auxin genes, they would be detected and could be subsequently subjected to current molecular biological analyses (cloning, sequencing etc.) thereby elucidating the genetic organisation in *R. phaseoli*, and would lead on to an understanding of the regulation of these genes and the enzymes that they encode. This approach has successfully shown significant homology between the auxin genes of *P. savastanoi* and those of *Agrobacterium tumefaciens* (Yamada *et al.*, 1985) and could used to determine whether genes similar to those of the IAAm biosynthetic pathway are present in *Rhizobium.*

5.5. Metabolism of tryptophan and IAA by detached roots and nodules of *Phaseolus vulgaris*

5.5.1. [^{3}H]-tryptophan feed to detached roots and nodules of *P. vulgaris*

Metabolism of labelled tryptophan by detached roots and nodules of *Phaseolus vulgaris* (cv. Canadian Wonder), revealed its conversion to ILA, IEt, IAld, IAA, IM and possibly ICA in nodule tissue (**Fig 3.7.1.**). In contrast, no indolic products were identified from the metabolism of tryptophan by the root tissue (**Fig. 3.7.3.**). This was perhaps due to tryptophan in the root being used preferentially for the production of compounds other

than indoles e.g. for protein synthesis, or that the root is incapable of IAA synthesis. Alternatively, the differences might be due to differential label uptake by these tissues. The spectrum of labelled metabolites detected was identical to those secreted by the bacterium *in vitro* and is suggestive that the pathway of IAA synthesis in *Phaseolus vulgaris* nodules is via IPyA and IAAld and thus identical to that proposed to operate in *R. phaseoli in vitro* (**Fig 5.1.**). Detection of indoles by TLC in *Lupinus luteus* root and nodule extracts (Dullaart, 1970) revealed the presence of two indoles, ICA and IGA, which were not detected in this project, in addition to IAA, IAld, and ILA which were.

5.5.2. [^{14}C]-IAA feed to detached roots and nodules of *P. vulgaris*

The addition of labelled IAA to root and nodule sections resulted in the detection of labelled IM and IAld in both root (**Fig. 3.8.2.**) and nodule sections (**Fig. 3.8.1.**) although the incubation period needed for detection of the compounds was four times longer in the nodule tissue (24 h) than in the root tissue (6 h). This is perhaps a reflection of a larger pool of endogenous IAA in the nodule (Badenoch-Jones *et al.*, 1984).

IAAsp was not detected as an IAA metabolite in the roots and nodules of *Phaseolus vulgaris* in this thesis and therefore the pathway of IAA catabolism in *Phaseolus* nodules appears to be different from that of *Pisum sativum* where formation of the IAA conjugate, IAAsp is the main route of IAA degradation in roots and nodules (Badenoch-Jones *et al.*, 1984). The detection of IAld and IM as IAA catabolites suggest that the major pathway of IAA catabolism in *P. vulgaris* roots and nodules is identical to IAA catabolism by the bacterium *in vitro*, that is involving the sequential decarboxylative oxidation of IAA to IM, IAld and ICA (**Fig. 5.2.**). However, although the steps from IAA to IM and IAld have been shown, the step from IAld to ICA remains speculative as ICA was not identified as an IAA metabolite in either roots or nodules.

5.5.3. Conclusions of IAA metabolic studies in *P. vulgaris*

The results presented suggest that while tryptophan is not converted by root tissue into IAA, the tissue appears to be able to catabolise IAA. It is therefore possible that root tissue *in vivo* does not synthesise IAA, but is capable of catabolising imported IAA. This result is in agreement with what is known about the sites of synthesis and IAA transport within the plant. IAA is primarily produced in leaf primordia and young leaves, not in the roots (Davies, 1988) and is transported basipetally from apical to basal regions (Rubery, 1988).

It was hoped that a comparison of IAA metabolism between the micro- and macrosymbiont would reveal differences in metabolic products which could then be used as 'markers' for IAA production by the bacterium in the symbiotic state (Ernstsen *et al.*, 1987). However, differentiation of the bacterial and plant IAA contributions to the nodule was impossible as there were no apparent differences in IAA metabolism, although clearly IAA production by the bacterium *in vitro* may differ from production by the bacteroid, the nitrogen-fixing form of the bacterium. Although not specifically studied here, it should be noted that IAA synthesis by *Bradyrhizobium japonicum* bacteroids isolated from the nodules of *Glycine max* has been detected (Hunter, W. J., 1989).

5.6. Altered IAA accumulation in Tn5 mutants

5.6.1. Possible reasons for altered IAA accumulation by mutants

There are a number of possible reasons for the observed alterations in IAA accumulation in the culture medium of the mutants produced by transposon mutagenesis (**Table 4.5.** and **4.6.**). Firstly, the Tn5 insertion may have affected genes directly responsible for tryptophan or IAA metabolism/regulation or secondly, have indirectly affected accumulation by disruption of the transport systems in and out of the cell.

The increased accumulation of IAA observed in the culture medium of overproducing mutants could be due to the transposon disrupting a gene responsible for regulation of auxin production in the bacterium. IAA biosynthesis could be rendered insensitive to feedback regulation by disruption of a repressor gene leading to abnormally high levels of IAA. It is also possible that the gene affected by the transposon is responsible for some part of IAA catabolism and therefore the high levels of IAA could be attributed not to overproduction, but to a build up of IAA through disruption of catabolic mechanisms. Apart from alterations in metabolism, increased secretion from the cell could account for the increased levels of IAA in the culture medium relative to the wild-type. Conversely, decreased IAA accumulation in mutant bacteria relative to the wild-type may be due to a decreased rate of biosynthesis, an increased rate of catabolism or to a combination of these. Alternatively, disruption of the cell transport systems could lead to decreased tryptophan uptake or decreased IAA secretion resulting in a decrease in IAA accumulation.

Questions concerning the rates of IAA biosynthesis and catabolism by mutants could be resolved by following the metabolism of radiolabelled tryptophan and IAA respectively. In addition, deficiencies in the transport system of the IAA-underproducing mutants could be detected by following the uptake of [^{14}C]-labelled tryptophan and its incorporation by the cell into IAA. The results of the latter experiment would need to be analysed carefully as it is likely that systems for tryptophan uptake and IAA excretion would be affected separately.

5.6.2. Comparison of IAA accumulation with other under- and overproducing mutants

The level of IAA, as quantified by HPLC, which accumulated in the culture medium of Tn5 mutants varied significantly from wild-type accumulation. IAA accumulation per litre of wild-type culture medium was up to 4.3-fold lower than the overproducing

mutants, while accumulation in the culture medium of the underproducing mutants was up to 100-fold lower than the wild-type (**Fig. 4.5.** and **4.6.**). The decrease in accumulation by low IAA-producing mutants was greater than that observed by Bakanchikova and Lobanok (1981), who isolated 10 underproducing mutants with decreases in IAA accumulation between 1.5- and 6.0-fold in comparison with wild-type *R. leguminosarum* strain L4. Despite the addition of identical amounts of tryptophan (200 mg l^{-1}) to the medium, IAA accumulation was approximately 10 times higher in the study of Bakanchikova and Lobanok (1981) where 21 μg ml^{-1} and 4 μg ml^{-1} of IAA were detected by colorimetric assay in the medium of the wild-type and the lowest IAA producing mutant respectively, compared with 2.2 μg, 9.6 μg and 0.023 μg ml^{-1} IAA in the medium of the wild-type, mutant H and mutant L respectively, in the current study. These differences might be due to different rates of accumulation between rhizobial species or to the different methods of IAA quantification used: colorimetric assay using Salkowski reagent in the Bakanchikova study and HPLC in the current work. Possible reasons for over-estimation of IAA content using the Salkowski reagent are given in **5.7.**

Although low IAA-producing mutants were generated in this study, a completely IAA-minus mutant of *R. phaseoli* was not detected. It could be that screening a larger number of colonies would lead to the isolation of such a mutant. IAA-minus mutants of *Pseudomonas savastanoi* (a non-diazotrophic bacterium) have been isolated (Smidt and Kosuge, 1978), but no IAA-minus mutant has been generated for any N_2-fixing bacterium*, for example, all 'IAA-negative' Tn5 *Azospirillum lipoferum* mutants isolated by Abdel-Salam and Klingmüller (1987) had residual IAA biosynthesis and accumulated

*Genotype I strains of *Bradyrhizobium japonicum* were identified as non-IAA producers in culture (Minamisawa and Fukai, 1991). These strains were however not tested for their nodulation ability.

IAA at between 8.6 and 55% of wild-type. As transposon insertion into a gene leads to a non-leaky mutation (de Bruijn and Lupski, 1984), Abdel-Salem and Klingmüller (1987) suggested that in *Azospirillum lipoferum* there are two pathways of IAA biosynthesis or alternatively, that there is more than one copy of the genes involved in one pathway and perhaps that, as has been suggested for oncogenic and non-oncogenic strains of *Agrobacterium tumefaciens* (Liu and Kado, 1979; Liu *et al.*, 1982), the genes for one pathway are borne on the chromosome while the genes for the other are carried on a plasmid. It has been further suggested that both the IPyA pathway and tryptamine pathway are present in *Azospirillum brasilense* (Hartmann *et al.*, 1983) and in *Agrobacterium tumefaciens* (Follin *et al.*, 1985). It appears possible that a similar situation may exist in *Rhizobium phaseoli* as it is known to carry plasmids (Beynon *et al.*, 1980). If this were the case, it would be extremely difficult to obtain a completely IAA-minus mutant as the function of at least two genes would have to be disrupted to knock out production entirely. There is also the possibility that the complete disruption of IAA production would be a lethal mutation as it is not known whether IAA is an essential requirement for the bacterium itself.

The high number of mutations found in the relatively low number of colonies screened, suggests that a large part of the chromosome is concerned with IAA production or with tryptophan uptake and IAA excretion from the cell. If, however, all of the observed mutations were associated with the same genes, it could be an indication that the transposon has a preferential insertion site within the genome of *Rhizobium.* Although it has been reported that Tn5 has low insertional specificity (Simon *et al.*, 1983; de Bruijn and Lupski, 1984), Abdel-Salem and Klingmüller (1987) and Shaw and Berg (1979) found a disproportionate number of mutations in genes affecting the biosynthesis of cysteine and methionine, which could indicate preferential transposon insertion into these genes. It is therefore possible that a similar high number of mutations are the result of preferential transposon insertion into the IAA biosynthetic genes.

5.7. Comparison of mutants designated high and low IAA-producers after Salkowski or HPLC testing

A number of colonies identified from Salkowski tests as overproducers, were subsequently re-classified as underproducers after analysis by HPLC (**Fig. 4.5.** and **4.6.**). Such anomalies could be due to differences in the physical environment to which the bacteria were exposed on solid and liquid media. Thus, while Salkowski reagent tests IAA accumulation around the colony as it grows on solid medium, HPLC analysis quantifies IAA accumulation in liquid culture. The IAA in the liquid medium is freely diffusible and readily available for degradation by the bacteria, while IAA produced on agar plates may diffuse away from the colony and thus be unavailable for biological degradation. This would have the effect of overestimating the amount of IAA produced by mutants on the plates.

Alternatively, differences could arise because the agar samples tested in this project were not cell-free and therefore damage to cells by Salkowski reagent (perchloric acid, **2.8.**) would result in IAA leaking out. The intensity of colour obtained would therefore be the sum of the extracellular and intracellular IAA and as such would tend to overestimate IAA production. This problem could be overcome in future work by taking a sample at the side of the colony where IAA can be detected but where there are no cells to affect the result.

5.8. Effect of mutants with altered IAA levels on infection and nodulation

To determine the effect of low/high rhizobial IAA accumulation on root infection and nodulation, the roots of *Phaseolus vulgaris* plants were inoculated with wild-type, high or low IAA-producing mutants.

In contrast to the wild-type where all inoculated plants became infected, only 50% of the plants inoculated with high and low IAA-producing bacteria became infected. This suggested that mutant bacteria with altered IAA production are less infective than the wild-type. Nevertheless, all plants which became infected by the mutant strains had nodules which were similar in size and number to those found on plants inoculated with the wild-type. The rate of nitrogen fixation, as determined by acetylene reduction, showed no significant difference between the high, the low and wild-type inoculated plants, and those plants infected by mutant strains showed no significant difference in nodule and plant biomass from the wild-type.

The results of the current work suggest that mutant bacteria with altered IAA production are less infective than the wild-type (**Table 4.8.**), which is in agreement with the work of El-Gamal (1985) who found that application of IAA higher or lower than an optimal to the roots of *Sesbania sesban* decreased the number of nodules formed. The reduced level of infection in plants inoculated with mutants may be an indication that a particular amount of IAA is required for nodulation and that increases or decreases disrupt recognition or acceptance by the host thus hindering infection at some stage during the infection process. The possibility also exists that this lowering of infectivity was due to further transposon insertions into the genome, thereby disrupting genes unrelated to IAA synthesis or regulation. However, this is unlikely as when *R. phaseoli* DNA was isolated, cut with EcoRI and hybridised to a probe for the Tn5, only one band was evident (**Fig. 4.12.**, lanes 4 and 6) indicating that there was only one insertion into the genome in each of the mutants examined (mutants L and H). However, this does not exclude the possibility that multiple insertions might have occurred during the culture of the mutant bacteria before root inoculation.

Hunter, W. J. (1987 and 1988) obtained contradictory results when he investigated the effects of high IAA producing mutants on nodulation. The majority of the high IAA-

producing *Bradyrhizobium japonicum* 5-methyltryptophan-resistant mutants investigated were poor symbiotic nitrogen fixers, and soybeans inoculated with these bacteria had a lower plant mass, nodule mass and fixed less nitrogen per gram of nodule than did plants inoculated with wild-type bacteria (Hunter, W. J., 1987). However, soybeans inoculated with a high IAA-producing mutant isolated a year later, had more nodules, more nodule mass, more total nitrogen and were larger than plants inoculated with wild-type bacteria (Hunter, 1988). This 'super nodulation' was also found by Kaneshiro and Kwolek (1985) who reported that *Bradyrhizobium japonicum* mutants which produced up to 30-fold more IAA than the parent strain and which unlike the parent strain, converted tryptophan to IPyA, stimulated nodulation of soybeans leading to increased root weight, nodule volume and acetylene-reducing activity, relative to plants infected with the wild-type. In contrast to these studies, there was no evidence in the current work of reduced nodulation (with the exception of levels of infection) or 'super-nodulation' of *Phaseolus* by the high IAA-producing mutant.

As was detailed in the introduction (**1.3.3.**), it has been recently discovered that a *nod* gene product from *R. meliloti* (Truchet *et al.*, 1991) is able to elicit the formation of genuine nodules on aseptically grown alfalfa. As a completely IAA minus mutant was not isolated in this thesis, it has not been possible to say with certainty whether rhizobial production of IAA also has a role to play in the infection/nodulation process or in the persistence of the nodule. The data presented, is in indirect agreement with the above authors in that the ability to accumulate IAA was not correlated with nodulation ability. Although all strains caused nodulation on the host plant, the mutant strains were apparently less infective than the wild-type strain. This lowering of infectivity could be due to, as was postulated by El-Gamal (1985) above, a required optimal level of IAA. Alternatively, it could be due to a change in the excretion of polysaccharides by both low

and high IAA-producing mutants, as there was a observed change in polysaccharide excretion from the cells of both mutants (**4.4.**), and perhaps this is responsible for the reduced levels of infection observed.

In a repeat of this experiment, it would be interesting to compare the actual amount of IAA in the wild-type nodules with those nodulated by the mutant strains to establish whether the bacteria under- and overproduce *in planta* as well as *in vitro*.

5.9. Tn5 stability in the absence of antibiotic

5.9.1. Loss of neomycin resistance from mutant strains re-isolated from *Phaseolus* nodules

As was discussed **5.8.**, while it is conceivable that differences in IAA production may not affect nodule biomass, an alternative explanation for the results obtained here could be that the transposon insertion is unstable and is lost from the bacterium during multiplication *in vivo*. That this may be the case is shown by the low percentage of resistant bacteria re-isolated from nodules of plants inoculated with mutants (**Table 4.11.**). It is possible that the bacteria responsible for nodulation of plants inoculated with mutant H and L are wild-type bacteria from cross-contamination. Clearly, the possibility of this cannot be ruled out, although, as was discussed in **4.6.**, considerable precautions were taken to avoid cross-infection between strains (**2.3.1.** and **2.3.2.**). However, the probability exists that the nodule is colonised by a mixed population of neomycin (+) and (-) strains as the bacterium multiplies during nodule development and that resistance was lost at this stage due to lack of antibiotic pressure.

5.9.2. IAA accumulation in the culture medium of re-isolated mutant bacteria

A representative sample of colonies, re-isolated from the nodules of *Phaseolus vulgaris* inoculated with wild-type, high or low IAA-producing mutants, were taken from Nm (-) plates i.e. a mixture of neomycin resistant and sensitive colonies, and chosen for the investigation of IAA analysis. Accumulation of IAA in tryptophan (+) medium by these bacteria revealed that there were no significant differences in IAA accumulation in the culture medium of these strains (**Table 4.12.**). These results suggest two possibilities. Firstly, that the wild-type strain was responsible for nodulation of plants inoculated with mutants. This possibility has been largely rejected in the previous section (**5.9.1.**). The second possibility is that the mutant bacteria have reverted to the wild-type state in the absence of antibiotic (Abdel-Salem and Klingmüller, 1987).

The Nm sensitivity and return to levels of IAA production similar to the wild-type suggest the transposon had been lost from the genome and the mutant strains have reverted. It is known that cell multiplication in the absence of antibiotic pressure (for example, in the rhizosphere and inside the root itself), may lead to daughter cells not receiving a copy of the transposon as constant pressure is needed to ensure that the Tn5 is stably maintained within the population (de Bruijn and Lupski, 1984). However, even in the presence of antibiotic, reversion can occur. A rate of 1.7 to 17.2 x 10^{-8} reversions per cell was calculated from the reversion of auxotrophic mutants to the prototrophic state in Tn5-induced mutants of *Azospirillum lipoferum* (Abdel-Salem and Klingmüller, 1987). All revertants were found to be Nm-sensitive and DNA hybridisation showed that all homologous sequences had been lost from the revertants suggesting excision and loss of the transposon. When strains harbouring Tn5-mutated plasmids are stored in the presence of antibiotic, 1-5% of the plasmid DNAs undergo DNA rearrangements (de Bruijn and Lupski, 1984). Storage of the bacteria as glycerinated cultures at -70°C reduces the frequency of rearrangements to below 1%, however, the frequency of rearrangement in actively growing bacteria in the soil or *in planta* for example, during

rhizobial multiplication inside the root, is unknown. It should be noted that loss of the transposon does not lead automatically to a return to normal function for the mutated gene as frequently there are segments of DNA left behind and deletions or rearrangements may have occurred at the site of insertion (de Bruijn and Lupski, 1984).

5.10. Conclusions of work concerning mutant strains of *R. phaseoli* 8002.

Tn5 mutagenesis proved to be a convenient and successful method of creating mutants which were easily selected by virtue of their antibiotic resistance. Studies on the IAA mutants produced revealed the potential of this system for the generation of bacteria with altered IAA levels. However, problems created by the possible loss of transposon highlighted the importance of creating a stable, permanently integrated transposon which can be used for *in vivo* experiments away from antibiotic pressure. Hopefully, with such a system, an IAA-minus mutant will be obtained which could be used *in planta* to provide answers to the many questions surrounding the physiology of the relationship between bacterial IAA synthesis and legume symbiosis.

CHAPTER 6

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Appendix A Media

1. Defined medium for *Rhizobium* (Modified from Vincent, 1970)

A (500 ml)		**g l^{-1}**
	$Na_2HPO_4.2H_2O$	1.0
	KH_2PO_4	1.0
B (470 ml)		
	NH_4Cl	0.5
	mannitol	10.0
	trace Elements (B & T)*	1 ml l^{-1}
	FeNaEDTA	0.01
	$MgSO_4.7H_2O$	0.25
	$CaCl_2.6H_2O$	0.1
Solid media:	Agar	15 g
C (30 ml)		**l^{-1}**
	pantothenic acid	1 mg
	biotin	1 mg
	thiamine	1 mg
	tryptophan	5-200 mg depending on experiment

*Baker and Torrey nutrient solution (**Nutrient solutions, Appendix B**).

Solution A and B were prepared separately, the pH adjusted to 6.8 and autoclaved at 120°C for 20 minutes. The solutions were left to cool to avoid precipating the phosphates before mixing and finally the vitamins (solution C) were filter sterilised into the medium.

Solid media: 15 g of agar was added to solution B after adjusting the pH. The solution was then autoclaved as before and left to cool to 48°C before the three solutions were mixed.

Antibiotic plates: The medium was autoclaved and cooled to 48°C before the antibiotic was added.

Differential medium (Modified from Bakanchikova and Lobanok, 1981): Medium was prepared as before, with the addition of 200 mg tryptophan filter sterilised into the media with the vitamins. These were dissolved in a larger volume of water and the volume of solution B adjusted accordingly.

2. Yeast mannitol agar (YMA)(Vincent, 1970)

	g l^{-1}
mannitol	10.0
K_2HPO_4	0.5
$MgSO_4$	0.2
NaCl	0.2
$CaCl_2$	0.2
$FeCl_3$	0.001
yeast extract	0.4
[agar	15]

All chemicals, except the agar, were mixed together in distilled water and the pH adjusted to 6.8 with H_2SO_4, the agar was added and the media autoclaved for 20 min at 120°C.

Antibiotic plates: As antibiotics are heat-sensitive, they were added to the agar after cooling to 48°C.

Congo Red plates (Hahn, 1966): Gloves and a mask should be worn when handling Congo Red as it is a known teratogenic agent. 990 ml of yeast mannitol agar was prepared and once cool, 10 ml of a 1/400 aqueous stock solution of Congo Red was filter sterilised into the medium, mixed well and the plates poured as normal. Colonies of *Rhizobium* absorb very little of this dye and so remain practically colourless, whereas many other bacteria take it up strongly (Hahn, 1966. Vincent, 1970). The dye is therefore of value when checking the purity of stocks and when isolating rhizobia from nodules.

Yeast mannitol agar slopes

Media was prepared and placed in a microwave for 3-4 min until the agar had melted. Then using a pipette or a syringe (minus needle), 10 ml aliquots of melted agar were dispensed into universal bottles and the caps screwed back on loosely. After autoclaving the bottles were inclined at an angle until the agar had set.

3. Luria-Bertani medium (LB broth)(Sambrook *et al.*, 1989)

	g l^{-1}
bacto-tryptone	10
bacto-yeast extract	5
NaCl	10
*Agar	15

The pH was adjusted to 7.5 with 5N NaOH and then autoclaved as before.

*When solid media was required, 15 g of agar was added before the pH was adjusted.

Appendix B Nutrient solutions

1. Baker and Torrey

A micronutrient solution for defined *Rhizobium* media. The components were dissolved in distilled water and stored at 4°C.

Component	g l-1
H_3BO_3	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.22
$CuSO_4.5H_2O$	0.08
$NaMoO_4.2H_2O$	0.025

2. Crones-N (Bond and Wheeler, 1980)

A major nutrient solution added to the Perlite/Vermiculite plant growth medium.

Component	gram
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

3. A-Z nutrient solution (Bond and Wheeler, 1980)

Minor nutrient solution added to the Perlite/Vermiculite plant growth medium.

Nutrient		g l^{-1}
boric acid	H_3BO_3	0.62
sodium silicate	$Na_2 SiO_3.5H_2O$	0.43
manganese chloride	$MnCl_2.4H_2O$	0.40
potassium permanganate	$KMnO_4$	0.40
copper sulphate	$CuSO_4.5H_2O$	0.055
zinc sulphate	$ZnSO_4.7H_2O$	0.055
aluminium sulphate	$Al_2(SO_4)_3$	0.055
nickel sulphate	$NiSO_4.6H_2O$	0.055
cobalt chloride	$CoCl_2.6H_2O$	0.055
titanium oxide	TiO_2	0.055
lithium sulphate	$LiSO_4.H_2O$	0.035
stannous chloride	$SnCl_2.2H_2O$	0.035
potassium iodide	KI	0.035
potassium bromide	KBr	0.035
sodium molybdate	$Na_2MoO_4.2H_2O$	0.03

Distilled water was added to bring the volume to 1 litre and the solution stored at 4°C.

Appendix C Solutions for indole extraction and HPLC

1. Potassium phosphate buffer (pH 8.0)

1. K_2HPO_4 174.18 g in 2 litre distilled water
2. KH_2PO_4 34.0 g in 500 ml distilled water

Solution 2 was added to solution 1 until the pH was 8.0. This phosphate buffer can be stored at 4°C as the potassium salt does not precipitate out under cold conditions.

2. Alpha-Floc pads

Alpha-floc, a cellulosic compound, was used to remove impurities during the extraction of indoles from *Phaseolus vulgaris* roots and nodules. The pads were prepared by placing a piece of filter paper in the bottom of a Buchner Filter. A small handful of alpha-floc was placed evenly on top and cleaned with approximately 1 litre of water under vacuum. Then the sample was poured on and washed through with the solvent.

3. Liquid scintillant cocktails

Liquid scintillant cocktails were prepared by mixing the constituent chemicals thoroughly before filtering into an amber glass bottle for storage.

(a) reversed-phase scintillant

PPO (diphenyloxazole)	20 g
distilled methanol	300 ml
Triton-X- 100	660 ml
distilled xylene	1,340 ml

(b) normal-phase scintillant

PPO	12 g
napthalene	150 g
Triton-X-100	50 ml
distilled toluene	1,000ml

Appendix D Molecular biology solutions

All solutions are from Maniatis *et al.* (1982) unless otherwise stated.

1. Preparation of phenol

Distilled phenol (Formachem, Strathaven, Scotland) was melted at 70-80°C in a water bath and 0.1% w/v 8-OH-quinoline (BDH Ltd., Poole, England) was added. Then 1 volume of TE buffer (pH 8.0) was added, mixed and once phase separation had occurred the upper phase was removed. This procedure was carried out a further three times. The buffer-saturated phenol was stored at 4°C in the dark. To prepare phenol-chloroform, one volume of chloroform was added to one volume of buffer-saturated phenol, mixed and any remaining buffer removed. Storage was as for phenol.

2. Buffers

TE (pH 8.0) working solution

10 mM Tris-Cl (pH 8.0)
1 mM EDTA (pH 8.0)

TAE (stock solution per litre)

	40x
Tris base (1.6 M)	193.6 g
Na acetate.3 H_2O (0.8 M)	108.9 g
EDTA-Na_2.2H_2O (40 mM)	15.2 g

The pH was adjusted to 7.2 with acetic acid then made up to 1 litre with water.

Membrane buffer - TE + 0.1 M NaCl [per litre]

10 ml 1M Tris-Cl (pH 8.0)

5 ml 0.2 M EDTA

5.8 g NaCl

SSC (stock solution per litre)

	20x
NaCl	175.3 g
Na citrate	88.2 g

The chemicals were dissolved in 800 ml water and the pH adjusted to 7.0 with a few drops of 10 M HCl. Volume made up to 1 litre.

SET (stock solution per litre)

	20x
NaCl	3 M
Tris-Cl (pH 7.8)	0.4 M
EDTA	20 mM

3. Antibiotic stock solutions

All antibiotics were from Sigma Ltd., Poole, England.

Ampicillin: 25 mg of the Na salt dissolved in 1 ml of distilled water.

Neomycin: 25 mg dissolved in 1ml of distilled water.

Rifampicin: 25 mg dissolved in 1 ml of distilled methanol.

All of the antibiotics were filter sterilised and stored in aliquots at -20°C.

4. Proteinase K (protease)

A stock solution of 1 mg ml^{-1} distilled water was prepared and stored at -20°C.

5. Denhardt's solution (Sambrook *et al.*, 1989 modified from Denhardt, 1966)

A stock solution of 100x Denhardt's solution (2% bovine serum albumen, 2% Ficoll, 2% polyvinylpyrrolidone) was prepared and stored at -20°C.

6. Denatured salmon sperm DNA

A stock solution of 1 mg ml^{-1} was prepared. Salmon sperm DNA was dissolved in water and sonicated to a length of 200-500 bp. The solution was then placed in a boiling water bath for 20 min. If necessary, water was added to bring the final concentration to 1 mg ml^{-1} and the liquid stored at -20°C.

7. Ethidium bromide

Stock solution: (10 mg ml^{-1}) ethidium bromide was dissolved in distilled water by stirring for several hours. The solution was transferred to a dark bottle and stored at 4°C. Working solution: 0.5 µg ml^{-1}.

8. Gel loading buffer (dye stop)

Dye stop was used at 6x buffer concentration and stored at 4°C.

0.25% bromophenol blue

0.25% xylene cyanol

30% glycerol in H_2O

1x TAE

9. Random priming reaction mix

Random priming solutions were prepared as follows:

Solution A: 1 ml of solution D (see below)

18 µl of ß-mercaptoethanol

5 µl of 100 mM of deoxynucleotides; dATP, dTTP and dGTP

Solution B: 2 M Hepes, adjusted to pH 6.6 with 4 M NaOH

Solution C: 5000 units of hexadeoxynucleotides were bought from Pharmacia and mixed with 556 µl of TE to give a final concentration of 9000 units ml^{-1}.

Solution D: 1.25 M Tris-Cl, pH 8.0

0.125 M $MgCl_2$

Reaction mix for 1 ml:

solution A	200 µl
solution B	500 µl
solution C	300 µl