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application of nitrogen-fixing systems in soil management

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PREFACE

In view of the continuing increase in cost and scarcity of mineral fertilizers resulting from the use of high-cost fossil energy, there is renewed interest in organic recycling and biological nitrogen-fixation to improve soil fertility and productivity.

FAO, in cooperation with the Swedish International Development Authority (SIDA) has assumed a leading role in this respect, including the sponsoring of a series of important international meetings to promote the use of organic materials and biofertilizers. Examples of these meetings include: the Expert Consultation on Organic Materials as Fertilizers held in Rome in 1974; the Workshop on Organic Recycling in Asia held in Bangkok in 1976 and the Workshop on Organic Materials and Soil Productivity in the Near East held in Alexandria in 1978.

The workshop in Alexandria recommended the further promotion of research, development, application and dissemination of information available on various aspects of biological nitrogen-fixation, including symbiotic systems of rhizobia/legume and Azolla/blue-green algae, and free-living nitrogen-fixing bacteria and blue-green algae.

Dr Y.A. Hamdi, a delegate and invited speaker at the workshop was requested to prepare the present Bulletin, which reviews the literature and provides information on the characteristics, production and use of biofertilizers in agriculture.

Although some of the methods described are being continually improved upon, it is hoped that the compilation of various aspects of nitrogen-fixation under one cover in this Bulletin will be of interest and assistance not only to research workers, but also to extension workers and planners concerned with the further development and refinement of these natural systems for soil improvement and management throughout the various agro-ecological zones of the world.

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l. INTRODUCTION

1.1 GENERAL

In order to help meet the excalating demand for food resulting from the dramatic expansion of the world's population, mineral fertilizers and pesticides have been used extensively to increase the yield of crops from arable land. However, intensive agricultural methods have introduced undesirable and sometimes catastrophic consequences by polluting air, soil and aquatic systems as well as foodstuffs. Furthermore a matter of concern is the high cost, in terms of fossil energy, of mineral fertilizers, especially those supplying nitrogen, and the wasting of fertilizers.

It is recognized that methods based on the combined use of chemical or physical factors and biological agents should improve soil production. However, chemicals applied in agriculture as nutrients, amendments, growth regulators, herbicides and disease control agents may alter soil and plant microorganisms favourably or unfavourably.

The microbiological activity in the rhizosphere is partially dependent upon climate and soil physical factors, and the latter can be manipulated to alter soil and plant microorganism associations. The prevention of harmful factors such as waterlogging, by good drainage, is a prerequisite for efficient nitrogen-fixing symbiosis between Rhizobium and legumes. Tillage methods affect many soil characteristics such as aeration, structure, temperature and water regime, all of which affect the microbiological balance.

Altering the rhizosphere flora by inoculation with certain organisms has long been recognized as a practical possibility. The beneficial effect of rhizobial inoculants on the yield and quality of crops and on soil fertility is widely accepted, as also mycorhizal inoculation, provided the proper environmental conditions are met. The use of other bacterial fertilizers, e.g. azotobacter and phosphate-dissolving bacteria, has been widely accepted in the Soviet Union and Eastern Europe.

Furthermore inoculation of non-legume crops by associative nitrogen-fixing Spirilla is promising. Algalization and application of *azolla-anabaena* systems have been evaluated and accepted for application as biofertilizers in India, the Philippines and Vietnam.

The purpose of this bulletin is to describe methods applied to utilize nitrogen-fixing systems in soil improvement and management and the consequent increases in crop yields obtainable by these methods. The rest of the integrated systems involved i.e. chemical and physical manipulations, are characterized with respect to their interaction with biological nitrogen-fixing systems.

1.2 GLOBAL STATUS OF NITROGEN

The most important element for all forms of life is nitrogen as it is found in nucleic acids, proteins and chlorophyll. Accordingly, all forms of live bacteria, fungi, green plants, and animals of all kinds cannot grow and function unless they aquire nitrogen in an acceptable form.

There is of course a vast amount of elemental nitrogen in gaseous form in the earth's atmosphere, dissolved in the oceans, and in both forms in soil and certain rocks. However, plants and animals cannot of themselves make use of elemental nitrogen. Thus gaseous nitrogen is always present in air spaces in plant tissues, but not utilized. Likewise animals inhale air and reject the nitrogen.

It is only in the combined forms that most organisms can utilize nitrogen; green plants grow satisfactorily if their roots are supplied with nitrate or ammonium salts and under natural conditions they can usually obtain these from the soil or from water if they are aquatics. Plants can synthesize enzymes and hence proteins and other complex nitrogenous compounds required for growth. Animals do not have this power and they acquire their nitrogen chiefly by consuming proteins originally synthesized by plants.

1.3 FIXED NITROGEN

Fixation of atmospheric nitrogen is the major factor in the nitrogen cycle. The natural processes though continuous are generally too slow in localized areas of intensive crop production. Physical fixation by lightening and rain, chemical fixation from volcanoes, natural or man-made smog, effluents from industry and mechanical transport and biological fixation has recently been estimated (Cast, 1976) and is presented in Table 1.

From these data it appears that the amount of nitrogen which is artificially fixed (i.e. man-fixed) is only about half of that fixed by natural means. Nevertheless artificial fixation of nitrogen causes a substantial modification of the nitrogen cycle in the natural ecosystem.

Table 1 ESTIMATES OF TOTAL NITROGEN FIXATION FOR THE YEAR 1974 (Cast, 1976)

Location or mechanism of fixation	Nitrogen fixed	
	kg/yr x 10	% of total
Agriculture	89	37.6
Legumes	35	14.8
Non-legumes	9	3.8
Grasslands	45	19.0
Forests	50	21.1
Unused land	10	4.2
Ocean	1	0.4
Atmosphere (lightening, etc)	10	4.2
Combustion	20	8.4
Industrial ammonium fixation	57	24.1
Use as fertilizers	39	16.5
Industrial uses	11	4.6
Inefficiencies (losses)	7	3.0
Total	237	100.0

With regard to natural fixation, grasslands comprise a major source of fixed nitrogen, some of which is derived from legume fixation. Non-legumes also contribute a substantial proportion of naturally fixed nitrogen.

1.4 FERTILIZER NITROGEN

The world production of nitrogen fertilizers (Table 2) shows a trend towards increased production, mainly in developed countries.

In Asia, the production is only half that of Europe, and of this, Japan contributes approximately a third of the total. Fertilizer production is low or nil in many regions of South East Asia and Africa.

For "bag" nitrogen i.e. industrially fixed nitrogen, it has been estimated that for one unit of nitrogen two units of oil are required. Thus if the cost of transport is included, the fertilizer nitrogen becomes too expensive especially for developing countries. In addition, environmental pollution resulting from the excessive use of fertilizer nitrogen, indicates that the greater exploitation of biological nitrogen-fixation is essential.

Table 2 WORLD NITROGEN FERTILIZER PRODUCTION (x 10^6 kg) [1]

REGION	YEARS			
	1974/ 1975	1975/ 1976	1976/ 1977	1977/ 1978
Europe				
Western Europe	10 180	4 316	9 540	10 085
Eastern Europe and U.S.S.R.	12 207	13 470	14 011	14 705
America				
North America	9 369	10 365	10 992	11 281
Latin America	1 198	1 225	1 283	1 359
Africa	181	144	147	150
Asia	3 340	3 432	4 237	5 074
Japan	2 341	1 557	1 171	1 446
All developed countries	34 605	35 235	36 414	38 202
All developing countries	7 908	8 611	9 814	11 408
Total World Production	42 514	43 847	46 225	49 611

[1] Source: FAO Fertilizer Yearbook 1978.

2. BIOLOGICAL NITROGEN-FIXATION

2.1 INTRODUCTION

The importance of legumes in building and conserving soil fertility has been recognized since the beginning of agriculture. With the development of new techniques for detecting the ability to fix nitrogen such as Kjeldahl, 15N and acetylene (C_2H_2) reduction methods, an increased number of species and systems able to fix nitrogen have been identified.

Such species and systems include free-living ones e.g. bacteria and blue-green algae; symbiotic systems e.g. rhizobia-legume, angiosperm-actinomycetes, and blue-green algae associations and associative systems e.g. rhizosphere, phyllosphere and lichens. All these systems have one characteristic in common: the ability to utilize nitrogen, but they differ in physiology, metabolic structure and genetic characteristics.

2.2 FREE-LIVING NITROGEN-FIXING ORGANISMS

2.2.1 Bacteria

The free-living bacteria known to fix nitrogen can be classified as in Table 3 (La Rue, 1977). The list contains organisms of soil, aquatic, phyllosphere and rhizosphere habitats, photosynthetic aerobic, anaerobic and facultative species. Collectively, they are capable of metabolizing a wide range of carbon compounds. Some can grow at pH 3 others at pH 10.

Table 3 NITROGEN-FIXING BACTERIA
(La Rue, 1977)

Family	Genera
Thiorhodaceae (Chromatiaceae)	Thiocapsa, Chromatium
Athiorhodaceae (Rhodospirillaceae)	Rhodospirillum, Rhodopseumonas
Hyphomicrobiaceae	Rhodomicrobium
Chlorobacteriaceae	Chlorobium
Spirillaceae	Desulfovibrio, Desulfotomaculum
Azotobacteriaceae	Azotobacter, Beijerinckii,Derxii
Entrobacteriaceae	Klebsiella,Escherichia,Enterobacter
Corynebacteriaceae	Corynebacterium
Bacillaceae	Bacillus, Clostridium

2.2.2 Blue-Green Algae

Nitrogen-fixing blue-green algae (Cyanophyceae) use light energy captured through photosynthetic processes to meet the energy requirements of nitrogen-fixation. They are the only organisms that exhibit a higher plant type of photosynthesis, i.e. oxygen production and carbon dioxide fixation, and have species capable of nitrogen-fixation. (See chapter 4).

2.3 ASSOCIATIVE SYMBIOSIS BETWEEN FREE-LIVING DIAZOTROPHS AND OTHER ORGANISMS

A great variety of associations exists between free-living diazotrophs and other organisms (Fig. 1) (Burns and Hardy, 1975). Although many such associations have been documented, there may still be some that exist unnoticed or overlooked.

2.3.1 Loose Associations

Many free-living microorganisms participate in what may be considered a loose association with other organisms. These do not involve structural or morphological accommodation of the nitrogen-fixing organism, but consist more of close physical contacts in which natural influence can be exerted between the symbionts.

i. Interaction among microorganisms

This type of symbiosis is difficult to define with precision because the Intensity and nature of the interaction is itself often subtle and transient. A consensus of the literature however, testifies to an enhancement in activity of free-living diazotrophs by microorganisms which do not fix nitrogen. For example organisms capable of decomposing polysaccharides have been observed to stimulate nitrogen-fixation by associated Azotobacter, Beijerinckia and Clostridium, possibly supplying carbon sources.

ii. Phyllosphere

The phyllosphere association involving free-living diazotrophs and aerial parts of higher plants have long been overlooked in nitrogen-fixation studies. Yet as early as 1956 it was shown that the leaves of tropical plants generally supported extensive surface populations of microorganisms, among which aerobic diazotrophs were prominent (Ruinen, 1975). Studies of tropical grasses show that these organisms exude biologically significant concentrations of carbohydrates, but little or no nitrogenous nutrients, thus providing a particularly suitable habitat for diazotrophs. These associations have also been indicated in temperate crops and have been shown to have the potential for a significant contribution to the nitrogen economy of temperate zone woodlands.

Ruinen (1975) also enumerated the common genera of microorganisms detected in the phyllosphere in different parts of the world e.g. Azotobacter, Beijerinckia, Derxia, Agrobacterium, Pseudomonas, Xanthomonas, Azotomonas, Mycoblana rubra, Spirillum, Myconostoc, Flavobacterium, Klebsiella, Aerobacter, Bacillus, Clostridium, Nocardia, Cyanophyceae, Chlorophyceae, lichens, bryophyta, and protozoa.

iii. Rhizosphere

The rhizosphere of various plants harbours high levels of nitrogen-fixing bacteria and evidence for significant nitrogenase activity has been reported for the rhizosphere of sugar cane, rice, grasses and marine angiosperms.

2.3.2 Associations With Animals

The scope of the participants in nitrogen-fixing associations has recently been extended beyond the limits of the plant kingdom. The involvement of higher animals is becoming increasingly well documented in reports of nitrogen-fixing activity, using the acetylene reduction technique, by the intestinal flora of both ruminants and non-ruminants. The animals implicated include swine, guinea pig, humans, reindeer, goat, sheep, steer and camel (Hardy et al, 1973).

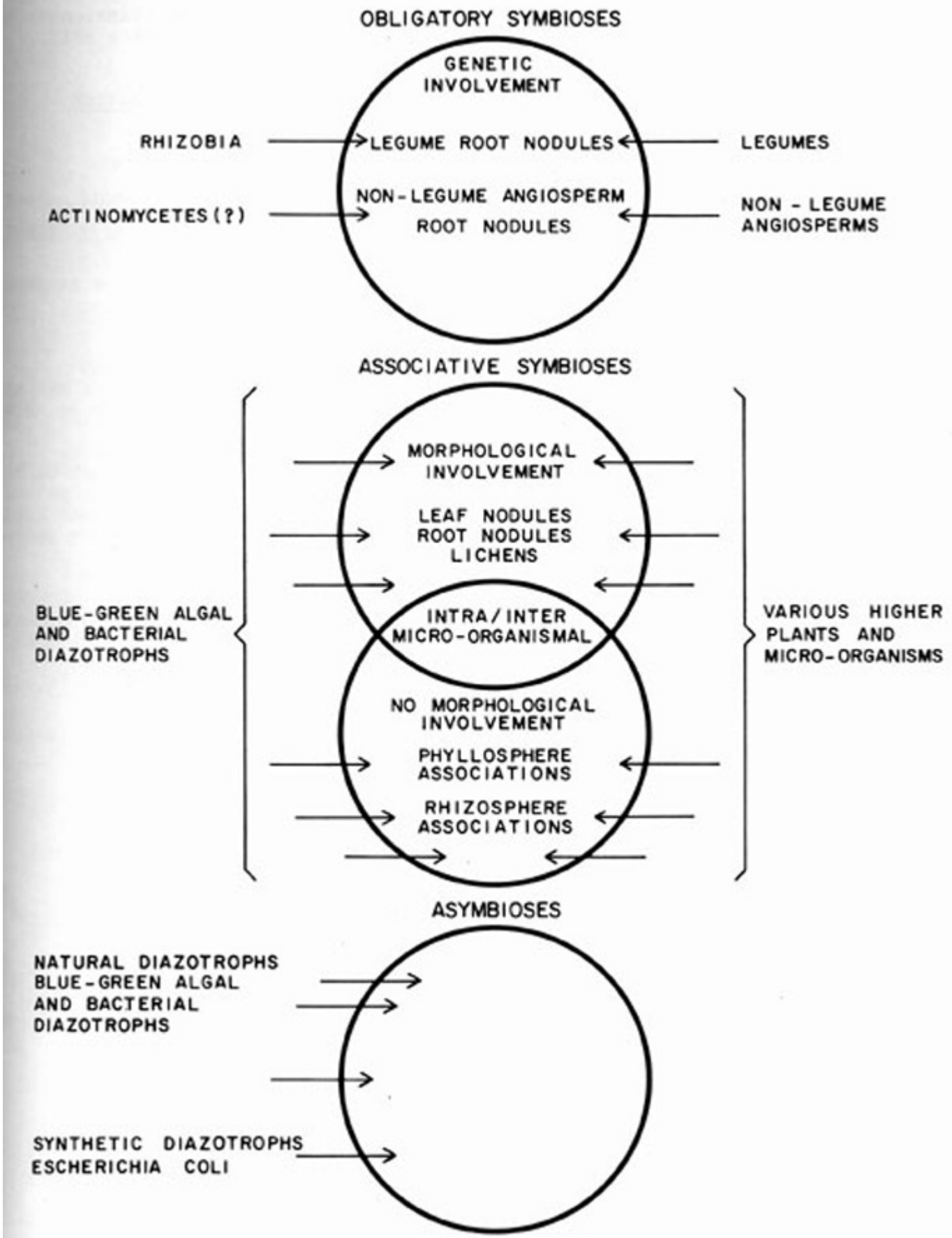


Fig. 1 Biological nitrogen-fixation relations (Burns and Hardy, 1975)

It appears that Klebsiella may be involved in the nitrogen-fixation found in human and swine faecal matter. However, possibly Escherichia coli and Enterobacter cloacae are also involved.

2.3.3 Associations Involving Morphological Modification Or Accommodation

i. Leaf nodules

In these nodules, a high concentration of nitrogen-fixing Klebsiella occurs extracellularly in the crude subepidermal leaf protuberances of tropical plants Pavetta, Psychotria, Ardisia and Dioscorea.

ii. Blue-green algae associations

Nitrogen-fixing blue-green algae are present in symbiotic association with fungi (lichens), several bryophytes (liverworts) and pteridophytes (water ferns), gymnosperms (cycads) and the angiosperm Gunnera.

The algal symbiont is always a Nostic or Anabaena species and, except for Gunnera, it is extracellular though often localized in morphologically specialized structures of the host plant. Moreover, the symbionts are often morphologically and physiologically distinct from the free-living species.

iii. Obligatory symbiosis

a. Non-legume angiosperms

These root nodule symbioses are sometimes referred to as an older type, and are the most studied member of the group. They share many characteristics with legume symbiosis but possess unique qualities which clearly warrant separate classification.

Symbioses are known among 14 genera of angiosperms, in 8 families and 7 orders. About one third of the more than 300 species are reported to be nodulated and probably nitrogen-fixing, but the actual fraction may be greater because many species have not been examined.

b. Legumes

The family Leguminosae comprises three subfamilies: Mimosoideae, Caesalpinoideae, and Papilionoideae with over 12 000 to 14 000 species. The exact incidence of nodulation and nitrogen-fixation is not known but reports indicate that about 90o/ of the subfamilies Mimosoideae and Papilionoideae and 33% of the Caesalpinoideae are nodulated.

These species are associated with bacteria of the genus Rhizobium and form characteristic nodules.

c. Tissue culture symbiosis

In an application of tissue culture technique to legume symbiosis, Holsten et al (1971) described conditions for effective rhizobia infection of callus tissue of soybean root origin. The resulting symbiosis provides a possible test system for investigating various facets of the legume symbiosis with minimal interference from plant influence.

The morphological changes which accompany the onset of nitrogenase activity in callus tissue were found to parallel closely the changes observed in intact nodule systems. Electron micrographs revealed the presence of a structure similar in appearance to an infection thread and also showed some of the intra-cellular bacteria to be enclosed in membranous sheaths or vesicles. With time, infected callus cells displayed the same dense population of microorganisms typical of intact nodules.

2.4 BIOCHEMISTRY OF BIOLOGICAL NITROGEN FIXATION

2.4.1 Nitrogenase Enzyme System

The nitrogen-fixing enzyme, nitrogenase, which reduces nitrogen to ammonia consists of two fractions:

i. the iron protein (Fe-protein or azo-ferrodoxin or component I),
ii. the iron-molybdenum protein (Fe-Mo-protein, or component II).

The two components catalyse nitrogen reduction only when combined and do not do so alone. It is possible to cross-react nitrogenase Fe-protein with Fe-Mo-protein from another organism to give active nitrogenase.

The Fe-protein is the smaller of the two and has a molecular weight of 50 000 - 70 000 and is extremely sensitive to inactivation by oxygen, losing over 70% of its activity after one minute of exposure to air. It is cold labile and is made up of two identified subunits, and contains 4 iron atoms and 4 acid labile sulphide groups, similar to ferrodoxin.

The Fe-Mo-protein is larger with a molecular weight of 100 000 to 300 000. It is less sensitive to oxygen than the Fe-protein. It is not cold labile but is composed of two subunits. The Fe-Mo-protein of A. vinelandii is crystallized.

2.4.2 Requirements For Nitrogen-Fixation

For nitrogen-fixation to occur the following requirements must be Satisfied:

- a nitrogenase enzyme system,
- a source of adenosive triphosphate (ATP),
- a source of reducing power,
- a protective system for the enzyme from oxygen inactivation
- the rapid removal of nitrogen fixed from the site of nitrogen-fixation to avoid inhibition of the nitrogenase.

3. RHIZOBIA-LEGUME SYMBIOSIS

3.1 THE MICROSYMBIONT RHIZOBIUM

The genus Rhizobium contains those bacteria able to form nodules on the roots of the family leguminosae. It also includes cultures which are no longer invasive, but which have an authentic history or origin from an invasive strain.

3.1.1 Taxonomy

In the 8th edition of Bergys' manual of determinative bacteriology Rhizobium is given as one of two genera making up the family Rhizobiaceae (Jordan and Allen, 1974). The second genus is Agrobacterium. Both genera produce cortical hypertrophies on plants. Rhizobium forms nodules mainly on roots of legumes and evidence is presented that rhizobia also produce nodules on non-legumes e.g. Trema and Zygophyllum. Agrobacterium produces disorganized galls on many kinds of plants.

The type species for the genus Rhizobium is R. leguminosarum Frank. Species are recognized on the basis of host preferences. The following species are recognized:

Species	Preferred hosts
Rhizobium leguminosarum	Pisum, Vicia, Lathyrus, Lens
R. trifolii	Trifolium
R. phaseoli	Phaseolous vulgaris, P. angustifolia
R. meliloti	Medicago, Melilotus, Trigonella
R. lupini	Lupinus, Ornithopus
R. japonicum	Glycine max

In addition many strains of rhizobia which associate with cowpea and lotus are now recognized as cowpea rhizobia and lotus rhizobia.

3.1.2 Morphology and Cytology of Rhizobium

Younger cells stain evenly with simple basic dyes (except that strains of R. leguminosarum and R. trifolii often contain metachromatic granules). Older cells generally take longer to absorb dye and have unstained areas of polyhydroxy butyrate (PHB) which give the cells a banded morphology

Young cells are motile with flagella which are either polar or peritrictious. Young rhizobia, while in culture media, are rod-shaped and become bacteroid under certain conditions, similar to the forms of rhizobia in the nodule.

Attempts to find heat resistant endospores among rhizobia, as claimed at times to exist, have not been successful.

3.1.3 Cultural Characteristics of Rhizobium

Much of the growth of most fast-growing rhizobia after 3-5 days consists of extracellular gum which may, in some cases, flow over the larger part of the agar surface. The growth of R. leguminosarum, R. trifolii and R. phaseoli is often clear as water or has differentiated into more opaque areas set in clear surroundings. Some are evenly mucoid throughout. Growth of R. meliloti is often more evenly opaque, even chalky white rather than clear or milky.

Colonies of slow growing strains do not become apparent for several days; they require about 10 days to reach maximum size and do not produce free-flowing gum. Pink pigmented rhizobia have been isolated from Lotononis bainesii.

The incorporation of congo red dye (at a concentration of 0.0025%) has been generally used (Vincent, 1970) as a differential media for rhizobia and contaminants including agrobacteria. Generally, rhizobia do not absorb the dye but the others do. But there are exceptions as some rhizobia strains absorb the dye and the extent of absorption may be affected by the nature of the medium and conditions of cultivation. Similarly, the addition of 0.0025% bromothymol-blue provides a useful indicator of relatively small changes in acidity or alkalinity.

Variation and dissociation in the colony type of rhizobia have been encountered. Variants of "R" and "S" forms have been found to be either invasive or not.

Yeast extract mannitol medium, generally known as medium 79 (Allen 1959), is suitable for growth of rhizobia. Most strains of rhizobia grow, poorly on glucose peptone agar but some, R. meliloti and Agrobacterium, make a reasonable degree of growth on this medium.

Growth of rhizobia in liquid media is generally evenly turbid and may become extremely viscous due to the production of gum.

Reactions in litmus milk have often been taken to be distinctive between R. meliloti (acid reaction) and the slightly alkaline reaction found with other species. The production of a serum zone appears as a strain specific rather than a species characteristic.

3.1.4 Physiology of Rhizobium

Detailed accounts of the microbiology (Vincent, 1977) and physiology (Bergesen,1978) of rhizobia are reported. A few points of practical significance are mentioned here.

i. Carbon source

In general, R. leguminosarum, R. trifolii, R. phaseoli and R. meliloti make good use of all carbon sources. The slow growers fail to use rhamnose, sucrose, trehalose, raffinose and dulcitol. Maltose, lactose and organic acids vary a good deal between strains.

ii. Nitrogen source

That rhizobia cannot utilize free nitrogen has been invalidated, as certain rhizobia strains fix nitrogen freely under defined conditions (Pagan et al,1975).

Many strains can utilize nitrate or ammonium as the sole or supplementary source of nitrogen but care should be taken to avoid a change in pH.

Strains unable to use inorganic combined nitrogen are generally satisfied by the addition of a single amino acid. In strains that differ in their ability to utilize certain amino acids, glutamic acid is generally acceptable.

About 5 - 10 ppm nitrogen are required for optimum growth of rhizobia; multiplication and respiration rates are augmented in media containing 108 ppm.

Urea is utilized by a large collection of representative strains and species, and most show a moderate degree of growth on biuret.

iii. Vitamin requirements.

Thirty five out of 63 representative rhizobial strains need the addition of one or more vitamins to the basal medium. The R. leguminosarum, R. trifolii and R. phaseoli group (26 out of 31 strains) require one or more of the vitamins biotin, thiamin and calcium pantothenate. When R. meliloti, R. lupini and cowpea rhizobia need vitamin (9 out of 23) they are satisfied with biotin. None of the R. japonicum strains tested (6 strains) required any vitamin (Graham, 1963 b).

Nicotinic acid, pyridoxin, folic acid, p-amino benzoic acid, inositol, B_{12} and riboflavine generally have no effect. Synthesis of certain vitamins has been confirmed, e.g. B_{12}.

iv. Mineral elements

Iron is essential for rhizobia. Low concentrations of salts are also beneficial.

Rhizobia generally require a total concentration of 0.5 m mol of calcium and magnesium. The distinctive effects due to a shortage of each ion become particularly apparent when levels of calcium are below 0.025 m mol or magnesium 0.1 µ mol. The viability of the culture is reduced when either element is deficient. Elongated and sometimes branched cells are developed in magnesium-deficient cells. Cells with low calcium content are irregular, swollen and roughly spherical (Vincent, 1977).

Strontium is a less efficient substitute for calcium for growth; neither barium nor magnesium induce growth.

The need for cobalt for growth has been confirmed: 0.01 m mol is required for maximum growth of R. japonicum. Cobalt deficiency prevents sufficient synthesis of vitamin B_{12} which is necessary for the normal function of methy-malonyl-Co-enzyme A mutase and leads to excessive production of ribonucleotide reductase apo-enzyme.

Low concentrations, e.g. 0.1-1.0 m mol of zinc and 0.1-1.0 m mol of manganese are required for rhizobia.

v. Oxygen

Graham (1963 a) indicated that none of the rhizobia tested grew anaerobically.
However, although excellent growth was obtained at less than 0.01 atm of oxygen, none grew without it at all.

vi. pH

In general all rhizobia grow within a pH range of 5.5-7.5. Acid tolerance in decreasing order, is (R. lupini, R. japonicum, cowpea) > (R.leguminosarum, R. trifolii , R. phaseoli) > (R. meliloti). Tolerance to high pH is in the reverse order.

vii. Temperature

From data available it appears rhizobia grow over the range 0-50°C with the optimum near 20-28°C. Among temperate strains, those of R. meliloti are the most tolerant (36.5-42.5°C), being 8°C higher on the average than those of R. leguminosarum and R. trifolii (31-38°C). In the collection of rhizobia from tropical legumes (cowpea miscellany) the range is from 30-42°C.

3.1.5 Ecology of Rhizobia in Soil

For the greater part of their existence, rhizobia survive as free-living bacteria in soil. They will survive and even grow in the absence of a host legume. A remarkable phenomenon occurs when the root of an appropriate legume is interposed into the environment of the free-living rhizobia. It is a phenomenon of mutual recognition between the two compatible partners. The legume plant root recognizes just the right kind of Rhizobium among all other bacteria, including other rhizobia in the vicinity, and the Rhizobium in turn recognizes just the right kind of legume root among all the other roots which may occur in that environment (Schmidt, 1978).

3.2 THE MACROSYMBIONT. THE LEGUMES

The family Leguminosae is a group of flowering plants found in both temperate and tropical zones. They range from small but widespread plants such as clover, through flowering plants such as lupins and bushes such as brooms, to shrubs and trees (e.g. Acacia). The family has been divided into 3 major subfamilies, the Papilionaceae, the Mimosaceae and Caesalpinaceae.

3.2.1 Cross-inoculation Groups

Groups of legume types that can exchange rhizobia with each other are known as cross-inoculation groups. Seven of these groups are now recognized. They are given below, with a list of the most important legumes in each.

i. Alfalfa group

Common name	Scientific name
Alfalfa	Medicago sativa
Button-clover	M. orbicularis
California bur-clover	M. denticulata
Spotted bur-clover	M. arabica
Black medic	M. lupulina
Snail bur-clover	M. scutellata
Tubercle bur-clover	M. tuberculata
Little bur-clover	M. minima
Tifton bur-clover	M. rigidula
Yellow alfalfa	M. falcata
White sweet clover	Melilotus alba
Huban sweet clover	M. alba annua
Yellow sweet clover	M. officinalis
Bitter clover (sour clover)	M. indica
Fenugreek	Trigonella foenumgraceum

ii. Clover group

Alsike clover	Trifolium hybridum
Crimson clover	T. incarnatum
Hop clover	T. agrarium
Small hop clover	T. dubium
Large hop clover	T. procumbens
Rabbit foot clover	T. arvense
Red clover	T. pratense
White clover	T. repens
Ladino clover	T. repens (giganteum)
Subclover	T. subterraneum
Strawberry clover	T. fragiferum

Berseem clover	T. alexandrinum
Cluster clover	T. glomeratum
Zigzag clover	T. medium
Ball clover	T. nigrescens
Persian clover	T. resupinatum
Carolina clover	T. carolinianum
Rose clover	T. hirtum
Buffalo clover	T. reflexum
Hungarian clover	T. pannonicum
Seaside clover	T. wormskjoldii
Lappa clover	T. lappaceum
Bigflower clover	T. michelianum
Puff clover	T. fucatum

iii. Pea and vetch group

Field pea	Pisum arvercse
Garden pea	P. sativum
Australian winter pea	P. sativum (var arvense)
Common vetch	Vicia sativa
Hairy or winter vetch	V. villosa
Horse or broad bean	V. faba
Narrow leaf vetch	V. angustiffolia
Purple vetch	V. atropurpurea
Monantha vetch	V. articulata
Sweet pea	Lathyrus odoratus
Rough pea	L. hirsutus
Tangier pea	L. tingitanus
Flat pea	L. sylvestris
Lentil	Lens culinaris (esculenta)

iv. Cowpea group

Cowpea	Vigna sinensis
Asparagus bean	V. sesquipedalis
Common lespedeza	Lespedeza striata
Korean lespedeza	L. stipulacea
Sericea lespedeza	L. cuneata
Slender bush clover	L. virginica
Striped crotalaria	Crotalaria mucronata
Sun crotalaria (Sunnhemp)	C. juncea
Winged crotalaria	C. sagittalis
Florida beggarweed	Desmodium tortuosum
Tick trefoil	D. illinoense
Hoary tickclover	D. canescens
Kudzu	Pueraria thunbergiana
Alyce clover	Alysicarpus vaginalis
(no common name)	Erythrina indica
Pigeon pea	Cajanus cajan (indicus)
Guar (cluster bean)	Cyamopsis tetragonoloba
Jackbean (horse bean)	Canavalia ensiformis
Groundnut (peanut)	Arachis hypogaea
Velvet bean	Stizolobium deeringianum
Lima bean	Phaseolus lunatus (macrocarpus)
Adzuki bean	P. angularis
Mat bean	P. aconitifolius
Mung bean	P. aureus
Tepary bean	P. acutifolius var latifolius

Acacia	Acacia linifolia
Kangaroo-horn	A. armata
Wild indigo	Baptisia tinctoria
Hairy indigo	Indigofera hirsuta
Partridge-pea	Chamaecrista fasciculata

v. Bean group

Garden bean, kidney bean, navy bean, pinto bean	Phaseolus vulgaris
Scarlet runner bean	P. coccineus (multiflorus)

vi. Lupine group

Blue lupine	Lupinus angustifolius
Yellow lupine	L. luteus
White lupine	L. albus
Washington lupine	L. polyphyllus
Sundial	L. perennis
Texas bluebonnet	L. subcarnosus
Serradella	Ornithopus sativus

vii. Soybean group

All varieties of soybean	Glycine max (Soja max)

There are certain legumes that appear to require specific strains of legume bacteria for effective inoculation. Some of these legumes are:

Common name	Scientific name
Birdsfoot trefoil	Lotus corniculatus
Big trefoil	L. uliginosus
Foxtail dalea	Dalea alopecuroides
Black locust	Robinia pseudoacacia
Trailing wild bean	Strophostyles helvola
Hemp sesbania	Sesbania exaltata
Kura clover	Trifolium ambiguum
Sanfoin	Onobrychis vulgaris (sativus)
Crown vetch	Coronilla varia
Siberian pea shrub	Caragana arborescens
Garbanzo (chick pea)	Cicer arietinum
Lead plant	Amorpha canescens

3.2.2 Nodules

Dart (1977) traced the recognition of nodules to drawings of legume roots in the sixteenth century, when Dalechamp (in 1586) remarked on the abundant nodules on Ornithopodium tuberosum.

i. Infection

b. Pre-infection stage

The point of chief interest and dispute is the nature and specificity of the curling reaction. Yao and Vincent (1969) distinguished degrees of curling and found that the characteristic shepherd's crook curling is only produced by bacteria able to infect the root, whereas indole-acetic acid causes a more generalized kind of deformation.

Some workers claim that polygalacturonase plays a role in the infection process, while others have questioned this premise. Small amounts of the enzyme produced by the host and detected in the rhizosphere are induced only by the homologous bacteria, or bacterial polysaccharide fractions, and this enzyme plays a part in infection.

Phytohaemagglutenins (or lectins) may be involved in the infection process. These substances are proteins with specific affinities for polysaccharides and with certain lytic properties, and they are commonly found in legume seeds. Those from soybean, agglutinate most soybean bacteria but not other rhizobia, and could therefore possibly function in the specific attachment of the bacterium to its host. However, the specificity of other legumes is not general. Some workers have claimed that lectins strip the bacterium of its polysaccharide envelop, thus allowing a closer association between host and bacterium.

Recently, the involvement of the polysaccharide callose in the infection process has been demonstrated. This B 1-3 linked glucan is known to be formed at the tips of growing root hairs and to be involved in some responses of plants to fungal invasion.

b. Zones of infection on roots

Infection of the legume root only occurs in certain transiently susceptible zones. Nutman (1962) indicated that infection is restricted at first to a few broad zones along the root, but areas of much denser infection occur within such zones. Fig. 2 shows the attachment of a R. trifolii cell onto a root hair.

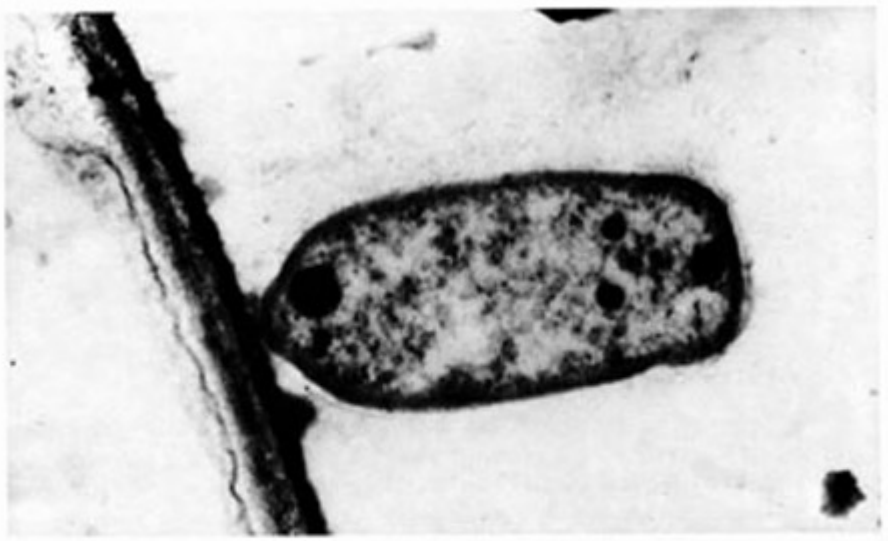


Fig. 2 *R. trifolii* on the root hair of clover (x 50 000)
(contributed by P.S. Nutman)

c. Infection threads

Infection of root hairs, following the initial penetration or invagination of the hair cell primary wall, proceeds by the development of a tube whose inner surface is lined with cellulose. It contains rhizobia lying end to end, usually in a polysaccharide matrix. The development of this tube, the infection thread, has been documented by time-lapse cinematography (Nutman, 1971). It can be regarded as an invagination of the root hairs and developing nodules.

Infection thread development in clover is associated with increased density and streaming. In about 4 hours the refractile infection thread sheath is visible and the root hair nucleus, now doubled in size and with a prominent nucleus, remains close to the tip of the growing thread. Should the nucleus move away from the tip of the thread it will cease growth and abort.

d. Nodule initiation

The infection thread penetrates through and between the root cortex cell towards the stele (Fig. 3). Actual nodule initiation, usually at the inner cortical tetrophoid cells, takes place near diploid cells in the pericycle that has the potentiality for lateral root formation. The close identity of nodule and lateral root initials is shown by common genetic control and parallel physiology. By changing root temperature or by delaying inoculation, nodules can be induced to form at positions that would otherwise form laterals. Nodules differ from roots in their requirement for some substances elaborated in the shoot, whereas roots have simpler needs. Nodules are integrated with the whole plant physiology and are therefore susceptible to changes affecting nutrition photosynthesis, translocation, photoperiodic and photochemical effects.

e. Nodule structure

Histologically, the term "mature nodule" refers to that stage when all tissues essential for nodule function are well defined structurally. Four distinct zones of tissue differentiation characterize the mature nodule. Figures 4 and 5 show cross-sections in nodules of Vicia faba and soybean.

- The nodule cortex: This tissue comprises the outermost four to ten cell layers of non-infected parenchyma. It is readily distinguished from the root cortex by the compactness and smaller size of the cells.

- The meristematic zone: This area constitutes the growing zone of the nodule. It consists of cells in a state of active division and growth. Within this region the process of tissue formation is continuous as long as the nodule is functional. All specialized tissues within the nodule originate from cell differentiation within the meristem.

  The position, size and duration of this area vary according to the host species. The relative position of the area determines the shape and to some extent the size of the nodule. Three types of nodule meristem are commonly recognized with respect to location:

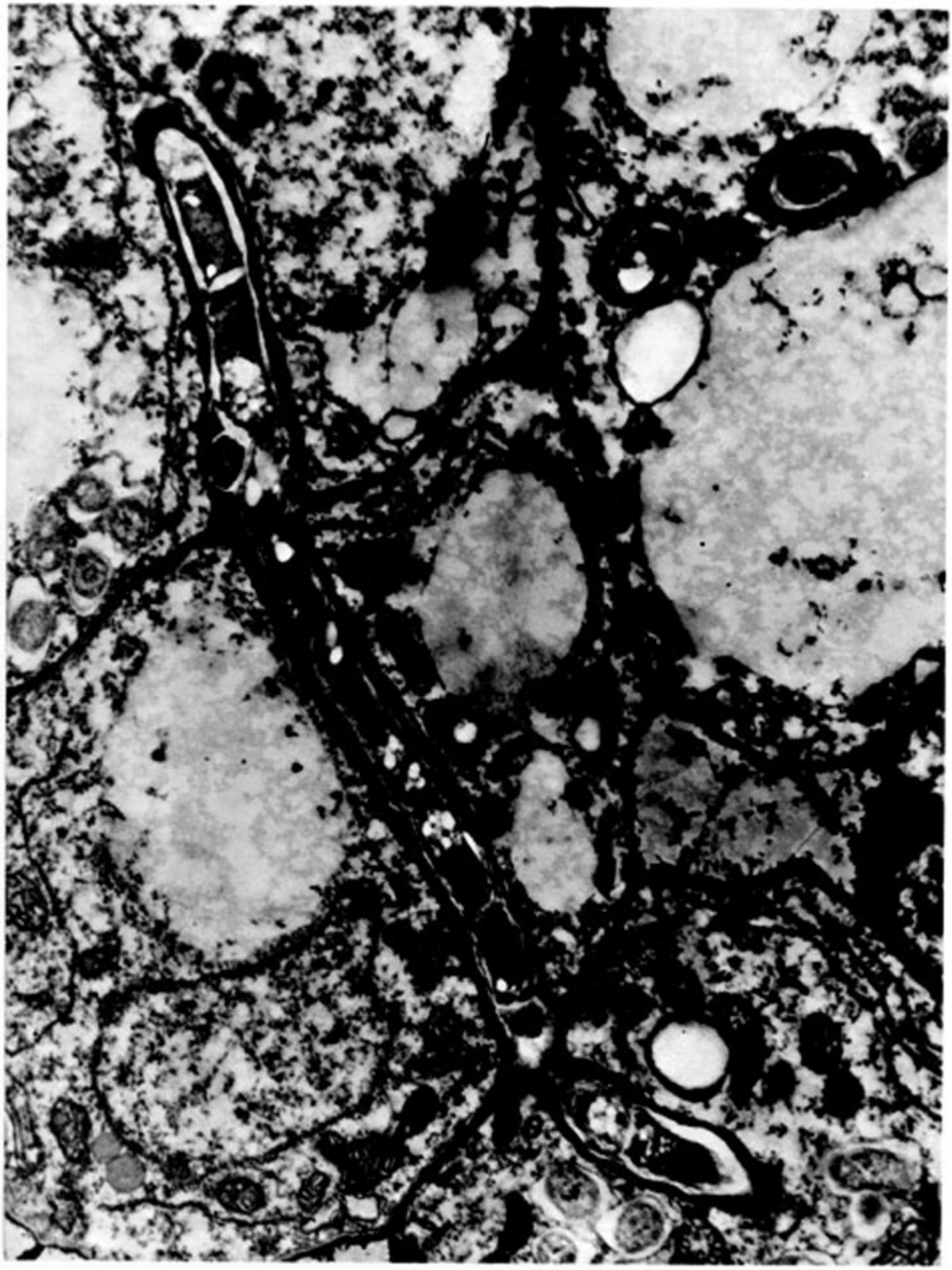


Fig. 3 Infection thread in cowpea nodule (x 17 500)
(contributed by P.S. Nutman)

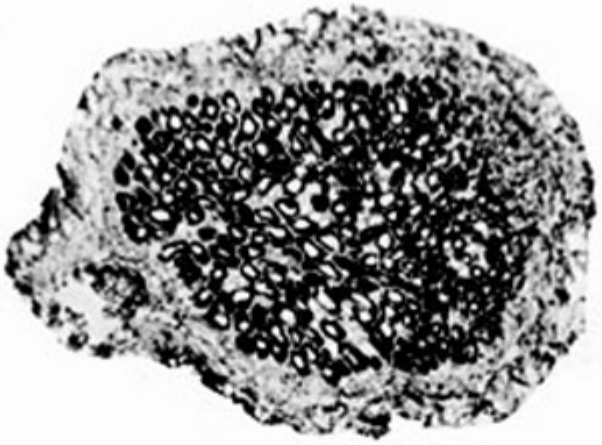


Fig. 4 Cross-section of *Vicia faba* nodule
(contributed by M. El-Mokadem)

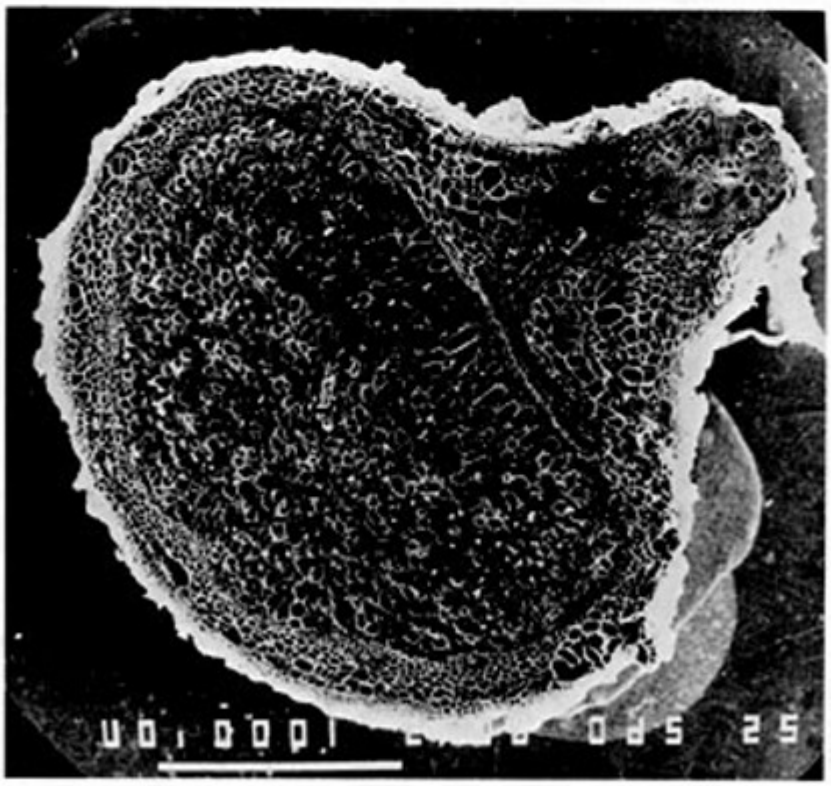


Fig. 5 Cross-section of soybean nodules showing vascular strands and infected tissue
(copyright J. Sprent)

(1) hemispherical: such as in bean, cowpea and soybean nodule where an equal rate of cell division throughout the peripheral area produces radial enlargement;

(2) apical: as in the elongated, irregular shaped nodules of alfalfa and clover;

(3) bilateral: exemplified by nodules on Lupinus spp wherein horizontal growth in opposite directions produces tissue hypertrophy which tends to girdle the root.

- The vascular system: This tissue serves for the transport of plant nutrients to the nodule, and, in turn, the translocation of nitrogenous substances and other metabolic by-products of the rhizobia to the host plant.

  Fully developed vascular bundles consist of xylem tracheids, phloem fibres, sieve tubes and companion cells enclosed in a parenchymatous sheath surrounded by a Casparian endodermis.

- The bacteroidal zone: This area, located in the central portion of the nodule, is literally the bacterial tissue. It is the site of the nitrogen-fixation process. Although non-infected cells are included, the term applies principally to the infected host cells, and stems from the fact that rhizobia therein often assume pleomorphic (bacteroidal) shapes (Fig. 6).

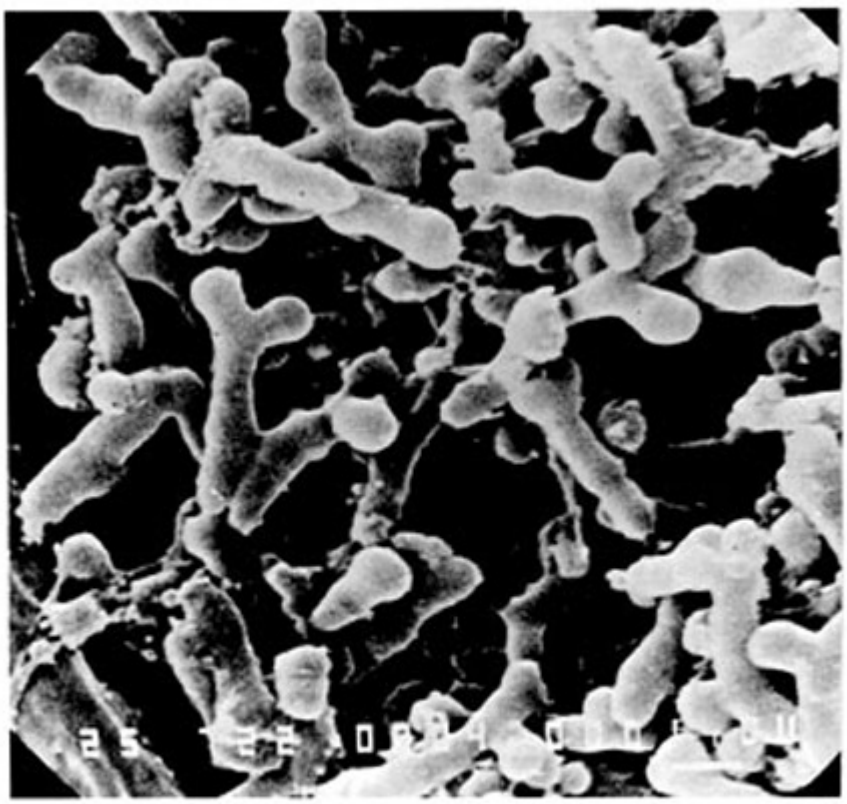


Fig. 6 Bacteroids of *Rhizobium leguminosarum* in *Vicia faba* cell (x 11 000)

3.3 GENETIC ASPECTS OF NODULATION AND NITROGEN-FIXATION BY LEGUME-RHIZOBIA ASSOCIATION

3.3.1 The Macrosymbiont. Legume Host

The host is involved in the processes of infection,nodule development and fixation. A conservative estimate is that there are 10 host genes in the plant involved in fixation and they are liable to mutate (Holl and La Rue, 1976).Most genetic analyses have been carried out on soybean (Glycine max), clovers (Trifolium spp) and pea (Pisum sativum).The genes rj1, rj2, rj3 and rj4 are known in soybeans. In red clover, genetic analysis showed the presence of the genes r, i1, ie, n and d. The genes Sym.1, Sym.2, Sym.3, no, and nod were identified in peas. These genes modify the response of host to rhizobia, ranging from reduction of efficienty to absence of nodulation.

3.3.2 The Microsymbiont. Rhizobium

i. Loss of symbiotic ability

a. Resistance to certain factors

Partial or full loss of symbiotic ability in some strains of rhizobia during growth in culture media has been observed by many investigators. The presence in the medium of inhibitory levels of amino acids has usually led to ineffective strains of rhizobia (Hamdi, 1968, 1969). The prevalence of ineffective clover-nodulating rhizobia in sandy acid soils has been ascribed to a build-up of ineffective mutants that are tolerent to metal ions of manganese, copper or aluminium (Holding and King, 1963).

Bacteriophages exert selective effects on variant types during growth of rhizobia, e.g. R. trifoli where a high portion of phage-resistant mutants of effective strains have been shown to be ineffective (Kleczkowska, 1971).

b. Antibiotic resistance

A streptomycin-resistant marker has been the one most frequently used in genetic or ecological experiments, or for biochemical study of associated blocks in symbiosis (Schwinghamer, 1977). Resistance to streptomycin occurs

independently of the change in symbiotic ability (Obaton, 1971).

c. Auxotrophy

Lorkiewicz et al (1971) noted a wide range of amino acid or vitamin requirements among auxotrophs isolated from an irradiated effective strain of R. trifioli, but all the auxotrophs were non-infective, parasitic or ineffective and all prototrophic revertants examined remained defective.

Abdel-Wahab (1977) showed that auxotrophic mutants of R. trifioli of histidine, leucine. lysine, adenine and uracil were ineffective (Fig. 7). However, auxotrophic mutants of arginine, methionine or tryptophane were effective.

ii. Genetic transfer within the genus Rhizobium

Several reports dealing with evidence of transformation, transduction conjugation,and plasmid transfer within the genus Rhizobium were reviewed by Schwinghamer (1977).

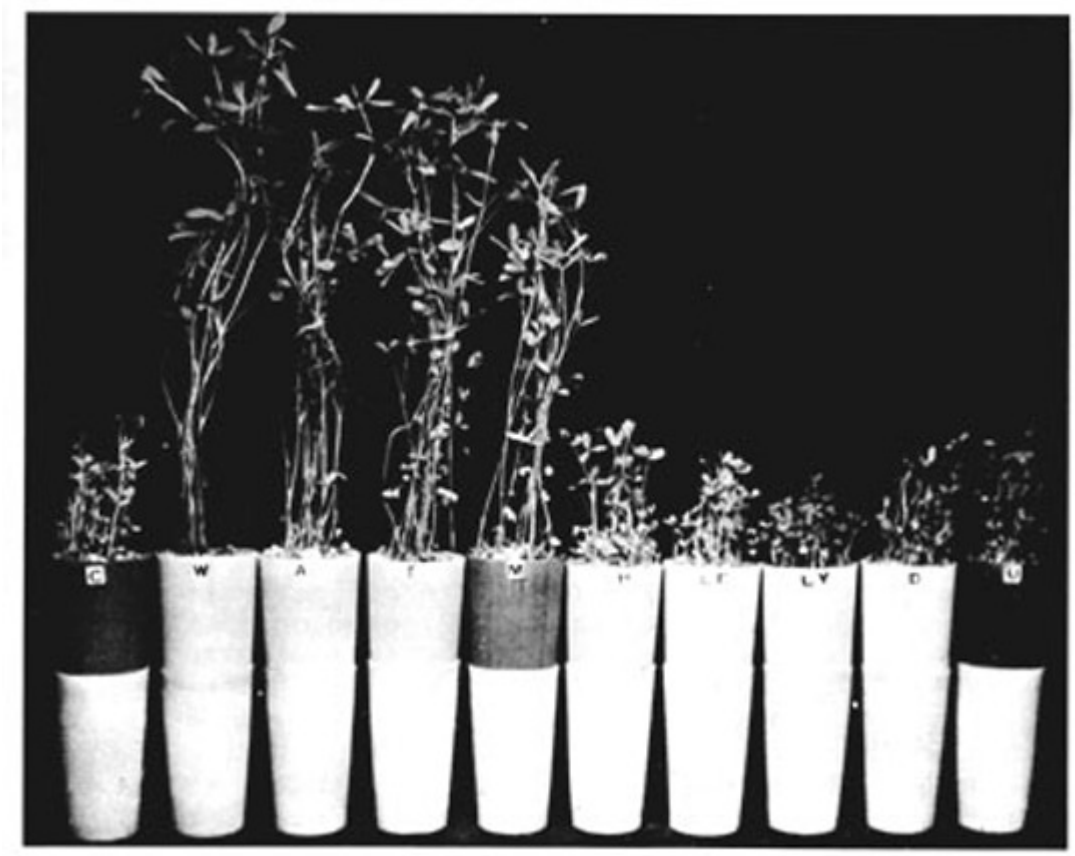


Fig. 7 Clover plants inoculated with single auxotrophic mutants of R. trifolii

C. control (non-inoculated).
W. inoculated with R. trifolii wild type strain
A. " " " Agrginine-less mutant
T. " " " Tryptophane-less mutant
M. " " " Methionine-less mutant
H. " " " Histidine-less mutant
Le. " " " Leucine-less mutant
Ly. " " " Lysine-less mutant
D. " " " Adenine-less mutant
U. " " " Uracil-less mutant
(contributed by S.M. Abdel-Wahab)

3.4 LEGUME INOCULATION

Inoculation is defined as the addition of effective rhizobia to leguminous seeds prior to planting for the purpose of promoting symbiotic nitrogen-fixation. Pure cultures on agar were first introduced in 1896 by Nobe and Hiltner (Date and Roughly, 1977), but later sterilized soil impregnated with rhizobia was used and then impregnated peat.

There is little doubt that inoculation is necessary in the majority of agricultural soils throughout the world. In particular inoculation is necessary under the following four conditions.

i. when a leguminous plant has not been previously cultivated;
ii. if the leguminous crop that was grown previously on the land lacked good nodulation;
iii. in a rotation cycle accompanying, or following, a non leguminous plant, and
iv. when the land has been abused or reclaimed from degraded conditions.

There are now six types of inoculants in general use: (1) moist peat powder, (2) liquid or broth, (3) agar or bottle culture, (4) oil dried rhizobia in vermiculite, (5) lyophilized rhizobia in talc, and (6) a granular type of peat inoculant.

3.4.1 Strain Selection

The selection of highly effective rhizobia strains is one of the principal obligations of the inoculant producer. A strain must be able to form effective nitrogen-fixing nodules on the hosts for which it is recommended under a wide range of field conditions. Other necessary characteristics include competitiveness in nodule formation, survival and multiplication in the soil, particularly in the absence of the host. Additional criteria include prompt and effective nodulation over a range of root temperatures; ability to grow well in culture; to grow and survive in peat and to survive on the seed; tolerance to pH and pesticides, and nodulation in the presence of combined nitrogen.

3.4.2 Collection of Strains

Date (1976) indicated two approaches to the collection of suitable strains of Rhizobium for new legumes or the establishment of a new collection of strains.

i. if the legume species is a new introduction, nodules should be collected at the same time as the plants so that microbiologists can isolate the Rhizobvum and subsequently provide it in the form of inoculum;
ii. uninoculated seed of the new species can be planted at a number of sites in the hope that native strains will form effective nodules from which suitable Rhizobium strains can be isolated.

3.4.3 Evaluation of Strains

Rhizobia for legume inoculants should be evaluated by:

i. screening for effectiveness in nitrogen-fixation;
ii. assay of the selected effective strain for nitrogen-fixing ability under field conditions, including competition for nodule sites, persistence and colonizing ability;
iii. testing for pH and pesticide tolerance, survival in culture and on seed.

For controlled environment and glasshouse conditions aseptic culture of plants in tubes or sand jars, as described by Vincent (1970), provides a satisfactory means of ranking strains for effectiveness. Undisturbed soil core samples (collected by driving an empty can into the ground) can also be used.

Field trials provide the ultimate test of a strain's ability, since the proportion of nodules formed and the amount of nitrogen fixed are the end result of many interacting factors. Effectiveness is measured either directly by determining the amount of nitrogen fixed or indirectly by measuring plant dry weight.

3.4.4 Bacteria Propagation

The submerged culture technique of growing rhizobia is now used by many producers of legume inoculants. Culture vessels or fermenters range from pyrex carboys to specially built tanks holding a few thousand litres of liquid medium. The starters are prepared as follows. Growth from 3-5 days of test tube cultures of the various strains is transferred into special flasks containing sterile yeast extract or mannitol agar medium. After 3-5 days incubation at 25°C, the starter flask is ready for use. The bacteria are washed from the surface and this suspension constitutes the seed or starter for transfer to a 45 litre (10 gallon) fermenter.

The fermentation medium for growth of rhizobia varies depending upon the species of rhizobia to be grown. Basically, it is a yeast extract or sucrose, and mineral salts medium. The medium is prepared and sterilized in the individual fermenters. After cooling to 27°C, the starter culture is added. Sterile air is introduced and dispersed into the bottom of the vessel through small perforations in a stainless steel sparger. The average fermentation cycle is 72 hours. At the end of this period the liquid culture usually contains approximately 5 000 x 10^6 cells/ml.

The liquid cultures from the 45 litre fermenters are used as starter or seed cultures for 1 800 litre fermenters. Again, 72 hours are required to attain the desired bacteria count of 5 000 x 10^6 cells/ml.

Fermenters vary from elaborate large ones (1 000-2 000 litres) to simple flasks or drums (10-100 litres). Good serviceable fermenters can be made from steel drums fitted with inlet and outlet tubes, inoculation and sampling ports (Fig. 8) (Date and Roughly, 1977).

3.4.5 Carriers of Rhizobia

The first artificial inoculants were liquid bacterial cultures added to seed or directly to soil. Although the results were frequently unsatisfactory, broth cultures of Rhizobium suspensions washed from agar medium continued to be used. However, critical comparisons of liquid cultures with peat inoculants consistently confirmed the superiority of the latter. Liquid cultures seem to lack the protection afforded by peat to rhizobia on seed following inoculation.

Many attempts ahve been made to produce inoculants other than peat based ones such as: 1) peat containing carbon black; 2) soil plus wood charcoal; 3) peat or soil ammended with materials such as lucerne meal, ground straw, yeast and sugar; 4) Nile silt supplemented with nutrients; 5) soil plus coir dust or soybean meal. Other materials have been tested as carriers of rhizobia: vermiculite, decomposed perlite and rice husk composts, ground rock phosphate, coffee husk compost; cob-earth (milled and decomposed), ground maize supplemented with nutrients; finely ground bagasse

which has been found suitable for soybean rhizobia; coal-based inoculant, (the best results were obtained with coal) bentonite, lucerne(CBL at 2:2:1) at a moisture content of 50%; and eat moss discs.

In spite of all attempts to substitute the peat inoculants they still remain the best carriers. Most of the other potential carriers have been studied in detail but they do not seem to possess superior properties to peat. Also some of the other materials are unavailable in many countries. However, it is clear that the search for new carriers has often been prompted by the lack of suitable local peat, and that the materials investigated were cheap and readily available in the areas concerned.

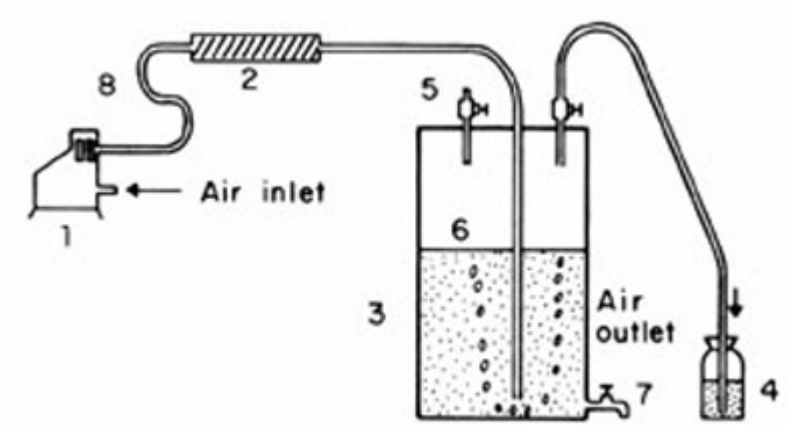


Fig. 8 Diagram of simple fermentation unit for production of rhizobial broth cultures (Date and Roughley, 1977)

1 Compressor for air supply. 2 Filter for sterilization.
3 Fermentation unit. 4 Outlet. 5 Inoculation port.
6 Broth stirred by air bubbles. 7 Sampling port.
8 Pressure reducing diaphragm.

i. Prior treatment of carriers

a. Source and character of peat

The most important factors to consider before selecting a particular peat as a carrier are the source and character of the material. Whereas the choice may be aided by chemical analysis, actual multiplications of Rhizobium strains in the peat are essential.

Most peat carriers have a high organic matter content but this is not an essential feature for good inoculant.

b. Drying of peat and particle size.

Drying of the harvested peat is an important step in its preparation. It has been shown, with Australia peat, that the final drying temperature should not exceed l00°C in order to avoid the formation of toxic degradation products and excessive rises in temperature when the peat is subsequently wetted by the addition of broth culture. Burton (1967) described the production of high quality inoculants by flash drying the peat carrier with hot air at 65°C to a moisture content of 7%. Dried peat is usually ground in a hammer mill. In Australia, the best results are obtained with peat carriers that pass through a 200 mesh sieve (Roughley, 1970). Whereas a small particle size is considered important by many inoculant producers, good results have been reported by Van Schreven (1970) with peat sieved through a 2 mm screen. Peat and soil carriers used by Indian workers, were ground to pass a 60 mesh sieve (Iswaran 1969)

c. pH and moisture content

Most peat deposits, with the exception of the Mount Gambier peats used in Australia, have a low pH and require the addition of a neutralizing agent before use. Peat carriers are usually adjusted to pH 6.0-7.0 with calcium carbonate. Calcium carbonate is better than sodium or potassium carbonates.

A final peat moisture content of 40-50% after adding rhizobia appears favourable for most peats used as carriers. Moisture contents below 30% or above 60% are generally unfavourable for survival. However, for sterile peat a 60% moisture content was recommended by Roughley (1970).

ii. Containers

The containers in which carriers are packed are important since their gas exchange and moisture properties may have a marked effect on the quality of inoculants. Bottles plugged with cotton wool and covered with cellophane have been used in Holland. Bags of low density polyethylene are used in Australia (0.038-051 mm gauge), while high density ones of 0.31-0.32 mm gauge are considered suitable for carriers in New Zealand and South Africa.

iii. Sterilization of peat

There is evidence that slow growing rhizobia, e.g. cowpea, soybean and lotononis may survive only poorly in a non-sterile peat that will never - theless support survival of fast growing strains (Roughley and Vincent, 1967). Whereas sterilization may not be essential for all peats, for some it is a pre-requisite in order to ensure success with various Rhizobium strains.

Steam is most commonly used for sterilization which requires 1-4 hours at about 120°C or 3 hours at 125°C. Dry heat can also be used for 1 hour at 120°C. (It should be noted however that Australian workers have found excessive heat treatment of peat detrimental to subsequent growth and survival of rhizobia).

Roughley and Vincent (1967) reported excellent survival of rhizobia in gamma-irradiated peat (5×10^6 rad) and this method is now used for all inoculants produced in Australia. However, gamma-irradiation does not appear to completely sterilize the peat and certain microorganisms seem remarkably resistant to doses up to $2.4\text{-}2.8 \times 10^6$ rad (Anellis et al, 1973).

Gaseous sterilization of peat has been tested in South Africa using Etox (90% ethylene oxide + l0% carbon dioxide) at 500, 750 and 1 000 mg/l for 8 and 16 hours respectively. A common characteristic of all Etox-treated peat is a marked decrease in number of rhizobia shortly after inoculation
(Strijdom and Deschodt, 1976).

3.4.6 Inoculant Preparation

The next step in the production of legume inoculants is to blend the liquid culture with the carrier medium. The freshly made inoculant is placed in shallow layers and cured at room temperature for several hours before packing. This dissipates heat and allows more intimate wetting. Superficial drying of the peat lessens the risk of mould growth, but if the peat is to be held longer than a few days at this stage, it should be covered loosely with polyethylene sheeting or transferred to large polyethylene bags or covered containers until packed.

3.4.7 Standards of Inoculants

The relationship between the number of viable cells put on the seed and success in securing nodulation is not simple. When conditions are extremely favourable, the application of a few rhizobia can result in rapid colonization of the developing root and plenty of nodules. On the other hand when conditions are unfavourable or there are numerous other rhizobia already present, an extremely heavy inoculum may be necessary to achieve success.

Table 4 illustrates accepted standards for different inoculants in Australia (Date and Roughley, 1977).

3.4.8 Multistrain Inoculants

Multistrain inoculants contain either strains from two inoculation groups, e.g. strains for clovers and medics, or a mixture of strains from the one group. The first type is used to simplify distribution but it is not recommended because of the reduced numbers of rhizobia of each host group. The second type is used to provide an effective inoculant for the wider range of host plants, to offset the dangers of undesirable variation resulting in loss of essential characteristics.

3.4.9 Tests for Control of Inoculants

Several tests are applied to control the quality of inoculants:

i. qualitative tests for immediate detection of contamination or wrong strain, in the broth culture (pH, agglutination, Gram smear, glucose, peptone agar and yeast mannitol agar);

ii. quantitative tests in broth: total and viable count;
iii. quantitative tests on peat culture: surface plate count on Congo red yeast
mannitol agar and plant infection counts.

Table 4 INOCULATION RATE AND MINIMUM NUMBER OF RHIZOBIA PER SEED FOR AUSTRALIA[1]
(Date and Roughley, 1977)

Host species	Seeds number/kg	Inoculation rate Kg seed/ 70g Peat[2]	Rhizobia minimum number/ seed[3]
Lotonis bainesii	3 840 000	5	110
Lotus pedunculatus	1 940 000	5	720
Trifolium repens	1 650 000	5	830
Stylosanthes humilis	440 000	5	3 180
Medicago sativa	430 000	10	1 640
Trifolium subterraneum	154 000	10	4 540
Macroptilium atropurpureum	88 000	10	8 000
Vigna sinensis	15 000	25	18 700
Glycine max	11 000	25	25 400
Lupinus angustifolius	7 500	25	37 400
Phaseolus vulgaris	4 000	25	70 000
Pisum sativum	3 100	25	90 000

1/ Inoculant Research and Control Service tested peat cultures.
2/ These values are higher than reported by Vincent (1970).
3/ For cultures in sterilized peat meeting the current standard at the end of the expiry period, 100 x 10^6 rhizobia/g, except for Lotononis culture which is 30 x 10^6/g.
For cultures in non-sterilized peat (min.standard expiry 30 x 10^6/g), the number of rhizobia/seed would be 10 fold fewer than listed.

3.4.10 Inoculant Application

There are two main methods of inoculant application: direct and indirect. Direct methods include putting inoculant into seed before sowing by dusting, slurry or pelleting. Indirect methods involve sowing seeds without inoculation and the inoculant is then applied into the soil in liquid or granular form.

i. Dusting

Manufacturers used to recommend that inoculant need only be mixed with the seed immediately before sowing. By this method some cultures adhere to the seed by lodging in the micropyle and in scratches and irregularities on the testa, and by electrostatic attraction. However, much of the inoculant tended to fall off especially when the seed was passed through machinery. Therefore dusting is no longer used.

ii. Slurry inoculation

By this method the culture is applied as a water suspension, or alternatively, the inoculant is mixed with moistened seed. However the seed must then be dried (not in direct sunlight), and while it is drying part of the inoculant tends to fall off. A large amount of the inoculant can be attached to seed by using an adhesive in the slurry, e.g. l0% sugar solution, gum arabic or cellofas A (carboxymethyl cellulose).

iii. Seed pelleting

Pelleting legume seed with lime provides a means to protect rhizobia from both acid soils and fertiizers. Pelleting involves mixing the inoculum with an adhesive, then putting the mixture onto the seed. The seeds are then rolled in a coating material. Adhesives usually used include synthetic or vegetable glues, gelatin, various sugars and honey. Whatever adhesive is used, it should have sufficient tenacity to prevent the coating material from sloughing off, which could lead to blockage in sowing machinery and also be detrimental to plant establishment. Coating materials tested include calcium carbonate in many forms, dolomite, various grades of gypsum, bentonite and other clay minerals, superphosphate, rock phosphate and other phosphorous compounds, titanium oxide, soil humus, talc and activated charcoal. The main requirements for a good general purpose pelleting material are that it should be relatively close to neutrality and be finely ground (90% at least should pass through a 300 mesh sieve according to Brockwell, 1977).

iv. Pre-inoculation

Seeds can be pre-inoculated by seed merchants prior to sale or Distribution to farmers. This implies a period of storage of the inoculated seed before it is sown.

A seed impregnation technique has been devised to extend the life of inoculum by introducing rhizobia beneath the seed coat where they are protected from drying and other adverse environmental conditions. Thompson, Brockwell and Roughley (1975) reviewed legume seed pre-inoculation in the USA and Australia, as a result of which they recommended that the date of preparation be displayed on all batches of pre-inoculated seed. From that date the expiry period of one month should be adhered to by all concerned.

v. Drenching method of inoculation (liquid inoculants)

Hely et al (1976) described a pressure controlled, commercial-scale, spray as a legume inoculation technique. It was developed for the accurate placement of suspensions of nodule bacteria into soil beneath seeds and for the application of greater amounts of inoculum than can be carried on seed inoculated according to the present practice.

vi. Granular inoculants

Fraser (1966, 1975) developed a granular type inoculant. Columnar beds of the granules together with a synthetic medium, were inoculated with R. meliloti or R. trifolii and allowed to incubate for 7 days. A mixture of milk and sucrose was then percolated through them, and after draining off the excess liquid they were spread out to dry at room temperature. Granules with counts ranging from 200 to 500 x 10^6/g are produced by this method, and storage tests indicate that at 20°C, a shelf life of two years is possible before the number of rhizobia falls below the required 300/seed. There are relatively few contaminants on the granules, and owing to the low moisture content they are unable to multiply during storage. In air tight containers, however, moisture accumulates on the surface of the granules and this allows moulds to develop. Comparative data on response of clover to the inoculant is presented in Table 5.

Table 5 RESPONSE OF CLOVER TO *R. TRIFOLII* AND GRANULATED INOCULANT (Fraser, 1966)

Method of inoculation	Storage	Rhizobia/seed applied	Rhizobia/seed present when sown	No. of plants	Foliage weight mg/plant	% plants with nodules
None		0	0	104	13.2	0
A. Agar culture in milk with 0.1% Ca phosphate	Freshly prepared	2 000	300	87	14.7	79
B. 1 g granule to 10 g seed	7 weeks	1 600	30	78	17.2	92

In another study, Aziz (1978) developed a granulated inoculant for R. phaseoli, using a pharmaceutical tablet coating machine, sodium carboxymethyl, cellulose and calcium sulphate. Response to the granulated inoculant was significantly higher than to other methods, e.g. seed pelleting.

Burton (1976) developed granulated inoculants from peat. With this method chemical treatments can be applied to the seeds without fear of killing the rhizobia. It is suitable for culturing soybeans in hot sandy soils or for aerial sowing of legumes.

3.4.11 Response to Inoculation

In short, the beneficial effects from inoculation are summarized as follows:

early nitrogen starvation is prevented; the demand by leguminous crops upon soil nitrogen is reduced; crop yield as measured in foliage or fruit is increased; the quality of the crop is improved; and finally, readily decomposable, rich, succulent green manure is made available for soil improvement.

Some examples of the response of different crop legumes to inoculation are given below.

i. Glycine max (Soybean)

Inoculation of soybeans with effective strains of R. japonicum may increase yields and seed protein percentage, when rhizobia are not already present. Kang et al (1975) showed that for maximum yield and to avoid stunting in early growth, the addition of 30 kg N/ha was necessary, even in well nodulated plants in Nigeria. Without inoculation, 60 kg/ha were required.

Various reports give the response of soybean to inoculation in Cameroon, Cuba, Egypt, Ghana, India, Iraq, Madagascar, Romania, Rwanda, Sierra Leone, Tanzania and Zaire. It is of interest to note that in Tanzania, Chowdhury (1975) found that although soybean had never been planted in certain fields near Morogoro, nodules were found on uninoculated plants. Nodulation was improved by inoculation but in some cases nodule weight was decreased. In another series of studies Ham, Lawn and Brun, (1976) showed that no significant response to inoculation was obtained when soybean was inoculated and planted in soils containing naturalized R. japonicum. Comparison of seed yields of non-nodulating isolines indicated that 20-41% of the yield of nodulating isolines was attributable to nodulation.

ii. Arachis hypogea (groundnut)

Dart (1978) indicated that Arachis hypogea has a great potential for nitrogen fixation. The following is the result of an investigation into the symbiotic characteristics of 48 groundnut germplasm entries 85 days from planting.

	Range
Nodule number	247 - 628
Nodule weight	0.3 to 0.75 g/plant
Nitrogenase activity	
mmol C_2H_4 plant n^{-1}	36 to 176
mmol C_2H_4 g/wt nodule n^{-1}	95 to 386

iii. Vigna unguiculata (cowpea)

Inoculation of cowpea is seldom necessary in the major growing areas, e.g. Western Nigeria, as there is usually an abundance of indigenous strains capable of good nodulation. The results of Kang et al (1975) support this view. However, other reports (Gargantini and Wutke, 1960) indicate that a well nodulated cowpea crop gained about 73 kg N/ha from nitrogen fixation.

iv. Phaseolus vulgaris (green bean)

These plants and especially their symbiotic associations are sensitive to water stress, soil acidity and many pests and diseases. The host has specific Rhizobium requirements and inoculation is often essential. Inoculation with appropriate rhizobia may double the yield and produce 15% more than crops provided with 50 kg N/ha (Drobereiner and Campelo, 1976).

v. Cicer arietinum (chickpea)

A significant response of Cicer arietinum to inoculation has been reported; for instance, increases in yield of up to 31% under rainfed conditions. However, in some locations, no significant response has been observed.

In the U.S.S.R. inoculation of chickpea has increased its areal mass by 25-36%. The nitrogen contribution from the symbiosis has been calculated to reach 41-60% for effective strains of rhizobia.

Brockwell and Gault (1972) evaluated the response of 21 lines of Cicer arietinum: all plant lines responded to inoculation with CB 1189; in terms of dry matter, 16 of the 21 responses were significant; on the basis of nitrogen content, all of the resonses except one, (line 19) were significant.

vi. Vicia faba (broad bean)

Broad bean showed a response (Table 6) to inoculation under Egyptian Field conditions (Taha et al, 1967).Figure 9 illustrates the type of Response obtained under greenhouse conditions.

Table 6 EFFECT OF INOCULATION IN PRESENCE OF SUPERPHOSPHATE ON BROAD BEAN YIELD AND THE TOTAL NITROGEN CONTENT OF THE SEEDS
(Taha *et al*, 1976)

Super-phosphate (kg/ha)	Mean yield (kg/ha)		Increase in yield (kg/ha)	Total nitrogen of seeds (mg/g)		Increase in nitrogen (mg/g)
	Uninoculated	Inoculated		Uninoculated	Inoculated	
0	1 310	1 550	240	62.9	65.4	2.5
117	1 572	1 716	144	64.3	68.5	4.2
233	1 716	1 856	140	64.9	72.4	7.5
356	1 786	1 856	70	65.2	70.7	5.5
466	1 642	1 893	351	65.4	73.4	8.0

Fig. 9 Response of broad bean to inoculation with *R. leguminosarum* strain 300-M (contributed by W. Gobreal)

vii. Lens culinaris (lentils)

Subba Rao (1976) indicated that inoculation of Lens culinaris increased the yield by over 70% in some locations, but not in others. Applications of P_2O_5 with or without inoculation significantly increased yield. In Egypt, Taha et al (1967) reported a significant response by lentils to inoculation.

viii. Desmodium

Under conditions in western Kenya, Desmodium uncinatum did not respond well to inoculation (Keya and Van Eijnatten, 1975). Seeds pelleted with lime, rock phosphate and gypsum were planted in potted field soil and in plots. In the pot experiment, inoculated seeds showed an initial advantage, but did not maintain it beyond 20 weeks. The field experiment showed no advantage from inoculation because of infection by indigenous, ineffective strains of rhizobia.

ix. Cajanus cajan

In field experiments conducted in 1967 at Pantnagar and in 1972 at Kanpur, neither Rhizobium inoculation nor application of nitrogen (40-80 kg/ha), P_2O_5 (50-150 kg/ha), or K_2O (25-50 kg/ha), affected yield whether individually or in combinations (Subba Rao, 1976). Pelleting with lime increased the yield significantly only at one location (Table 7).

x. Clover

Hastings and Drake (1972) isolated the rhizobia R. trifolii from five different New Zealand soils. Eleven strains of rhizobia were inoculated into white and subterranean clovers grown in the different soils in pot experiments. The results (Table 8) show that all strains were effective for red and white clover, the best strains being 2666, 2153, 2163 and 2641. With subterranean clover, strain 2121 was considerably better than any other strain. Figure 10 shows the type of response of berseem clover to inoculation under greenhouse conditions.

Table 7 GRAIN YIELD (kg/ha) OF CAJANUS CAJAN IN FIELD EXPERIMENTS AT DIFFERENT SITES IN INDIA, WINTER 1972-3 (Subba Rao, 1976)

	LOCATION			
Treatment	Hissar soil pH 8.0 Var. T-21	Badnopur soil ph 8.0 not reported	Bangolore soil pH 6.2 Var. S-5	Indhiana soil pH 7.6 Var. T-21
Uninoculated (control)	2 405	786	536	1 730
Rhizobium	2 847	882	569	1 720
Rhizobium + talc pelleting	3 030	861	639	1 410
Rhizobium + charcoal pelleting	2 638	852	549	1 510
Rhizobium + lime pelleting	2 880	849	511	2 010

Table 8 WEIGHT OF FOLIAGE (g) FROM PLANTS INOCULATED WITH SELECTED *RHIZOBIUM* STRAINS IN GLASSHOUSE TRIALS (TOTAL WEIGHT FROM FOUR REPLICATIONS IN EACH OF FIVE SOILS)
(Hastings and Drake, 1972)

Strain No.	White clover	Red clover	Total white + red clover	Subterranean clover	Total
2120	215	164	379	147	526
2121	228	174	402	300	702
2153	227	192	496[1]	120	589
2163	263	200	463[1]	45	508
2272	219	192	411	15	426
2273	237	184	421	45	466
2641	261	198	459	49	508
2643	208	170	378	101	479
2647	227	183	410	90	500
2666	257	217	474	100	574
2669	231	197	428	20	448
	33	29	62	20	

[1] Superior strain

Fig. 10 Response of berseem clover to inoculation with *R. trifolii* strain 203
(contributed by W. Gobreal)

3.4.12 Factors Affecting the Success of Inoculation

There are many factors which can interfere with the success of inoculation and hence nitrogen fixation. The amount of nitrogen fixed by the legume rhizobia symbiotic system is influenced by many chemical and physical factors of the environment which may affect the host plant, the Rhizobium and the development and effective functioning of nodules.

i. Soil atmosphere

The composition of the gas phase may differ considerably from that of the atmosphere, because there is more carbon dioxide and less oxygen in the soil due to respiration of microorganisms and plant roots.

ii. Atmospheric nitrogen

In general the concentration of nitrogen gas in the soil is Sufficient to allow nitrogen fixation to take place without limitation. Under waterlogged conditions, there will be a lower concentration of nitrogen gas and, at the same time, the oxygen level will be too low to permit normal plant growth.

iii. Oxygen

For the formation and functioning of nodules, a liberal supply of oxygen is necessary. In the soil, the oxygen level is usually well below 20% and at this level both nodule formation and functioning will be suboptimal. Moreover, when there is excessive water, there may be a temporary reduction in nitrogen-fixing activity because the thin layers of water surrounding the nodules will cause a reduction in the oxygen supply.

iv. Carbon dioxide

Wilson (1940) reported that an increase of carbon dioxide in the atmosphere stimulated nitrogen fixation by nodulated clover plants and this was attributed to an increase in photosynthesis. The beneficial effect on nodulation and nitrogen fixation of aerating legume roots growing in water cultures, with air containing 4% carbon dioxide was reported by Mulder and Van Veen (1960).

Hardy and Havelka (1973) showed that carbon dioxide enrichment for soybean increased nitrogen fixation more than five-fold by doubling nodule specific activity, doubling average nodule mass, and extending the exponential phase.

v. Soil pH

A great deal of variability in pH tolerance has been found among rhizobia. Vincent (1965) reviewed the effect of pH on rhizobia and concluded that R. meliloti is acid sensitive, R. trifolii is less so, and R. japonicum is acid tolerant (e.g. as low as pH 3.5).

vi Temperature

Temperature is one of the important factors affecting the survival of rhizobia in soil. R. meliloti strains are relatively tolerant to high temperatures and limiting ranges are reported to be between 36.5-42.5°C.

R. meliloti in peat has survived for 21 days at 40°C in the Laboratory and for longer periods at intermittently higher temperature in the field. Chowdhury et al (1968) indicated that temperatures higher than 35-40°C were lethal for both R. trifolii and R. lupini species grown in sterile unfertile soil. Salem and Szegi (1971) showed that optimum growth of R. phaseoli in three Hungarian soils was at 30°C, but incubation at 40°C limited its growth in all three soil types.

vii. Moisture

As is the case for all soil bacteria, rhizobia must be surrounded by a film of water in which the solutes are not so concentrated as to raise osmotic problems for the cell. Too little water (rather than too much) is a threat to their survival and function (Schmidt, 1978).

Foulds (1971) examined the survival of rhizobia during air drying of three soils by plant infection techniques. R. trifolii proved to be more tolerant of drought than R. meliloti and Rhizobium from the Lotus group.

Hamdi (1971) studied the movement of R. trifolii in sterilized soils. With suction applied to soils of varying textures, zones of movement were sharply decreased by increased water tension, and ceased when water-filled pores became discontinuous.

viii. Salinity

Several reports indicate that growth of rhizobia, and nitrogen fixation by various legumes, is reduced with increasing levels of salts (Pilai and Sen, 1966; Hamdi and Al-Tai, 1976). Steinborn and Roughley (1974) showed that the death rate of R. trifolii increased with increasing salt concentration in peat. An increase in the salt concentration in irrigation water led to a significant decrease in weight of soybean plant, pods and nodules (Ala el-Din, 1976).

Growth of soybean was totally inhibited at 0.8% or more of sodium Chloride and at 1.5% of sodium sulphate. However, at 0.1% of sodium sulphate a stimulation of growth was noted (Abd el-Gaffar 1976).

ix. Water and light stresses

Sprent (1976) examined the effect of water stress on nitrogen fixation by Trifolium repens, Glycine max, Vicia faba and Lupinus arboreus. Water stress affects the growth of young nodules, and the formation of new nodules (Table 9), but whether the reduction in nodule number resulted from few rhizobia being available for infection for bacteria or from effects on the infection process or both is not clear.

Shading by trees is a major factor affecting the nodulation and nitrogen fixation of the lupin, and its survival under a forest canopy (Sprent, 1976). Shading mainly reduces nodule number and size, but only the deepest shade appears to affect nodule activity per unit weight of tissue of P. vulgaris.

x. Seed coat diffusates

Toxic, water soluble, seed coat diffusates have been demonstrated to inhibit the growth of rhizobia. The seeds of several legumes prepared in this manner have shown considerable variation in their toxicity to various Rhizobium spp.

xi. Combined nitrogen

Forms of combined nitrogen are reported to decrease the nodulation of legumes and to reduce nitrogen fixation. The degree of inhibition depends on many factors including the concentration and form of nitrogen, the time of application and strains used.

Vigne et al (1977) observed that growing soybean hydroponically on urea allowed effective nodule development and function. This suggests that if this form of nitrogen could be stabilized, i.e. if urea hydrolysis and subsequent nitrification could be prevented, soybean could simultaneously fix nitrogen and absorb combined nitrogen.

Table 9 EFFECT OF WATER STRESS ON NODULE NUMBER, SIZE AND ACETYLENE REDUCING ACTIVITY OF P. VULGARIS PLANTS (44 DAYS) INOCULATED WITH TWO STRAINS OF R. PHASEOLI
(Sprent, 1976)

	Rhizobium strains			
	3601		3605	
	Control	Stress	Control	Stress
Pmoles C_2H_2/mg/min	16.45	1.75	37.45	3.15
Nodule number	28.40	8.3	18.50	4.5
Average nodule weight (mg)	1.44	0.95	1.72	1.16
Water content of sand (% DW)	6.54	0.71.	6.34	0.81

xii. Trace elements

Many disorders due to deficiencies of trace elements have been shown to affect the growth of legumes. Although most of these deficiencies do not specifically inhibit the fixation of nitrogen, they limit the growth of the host. A more specific role has been demonstrated for molybdenum, cobalt, and boron.

- Molybdenum
  The specific role of molybdenum in the efficient functioning of the formed nodule has been studied intensively. 10-15 ppm molybdenum appeared necessary for maximum nitrogen fixing ability in lucerne, 4 to 8 ppm sufficed subterranean clover.

- Cobalt
  The essentiality of cobalt for symbiotic nitrogen fixation has been demonstrated in soybean, lucerne and subterranean clover under controlled conditions.

- Boron
  Boron deficiencies that interfere with nodule function have been reported from time to time, establishing the role of this element, a deficiency of which affects the vascular system leading to the nodule and hence causes a shortage of carbohydrate at the fixation site.

xiii. Pesticides

All fungicides are likely to be toxic to rhizobia to some degree. Thus when the host has to be both treated against fungal infection and inoculated with rhizobia, a balance has to be achieved between the two effects, by the careful selection of fungicides. Compounds containing toxic metals such as mercury, copper and zinc, are always a serious risk, as also halogen substituted aromatic compounds, although reports differ as to the degree of risk in relation to formulations used.

Various fungicides based on thuram are generally the least toxic to rhizobia and this advantage has in fact been accepted for use in the field.

Insecticides have not been studied in any detail in the laboratory, but there are clear indications of toxic effects with inoculated seed.

Some herbicides appear to have no effect on rhizobia, while some are harmful. Information on inhibiting levels is confused by the fact that species, and strains within species, differ considerably in their sensitivity to even one herbicide, and because results are likely to be affected by local conditions and time of inoculation.

xiv. Competition

One important attribute of a given strain of Rhizobium is its ability to compete in nodule formation with closely related soil rhizobia. The ability to colonize the rhizosphere in high numbers is essential in all instances.

An example of the results of studies on this subject is the analysis of the competitive ability of R. japonicum strains 110, 38 and 76 introduced into rhizobia-free soil. Strain 110 produced 98% of the nodules when applied with strain 38, and 76% when combined with strain 76. Strain 76 produced 95% of the nodules when combined with strain 38 (Caldwell, 1969).

xv. Biotic factors

Many hypotheses have been advanced to account for the failure of rhizobia to colonize readily or for the decline of the populations naturally present or those deliberately added to soils. The hazards to colonization include toxin-producing microorganisms, protozoa and nematodes.

Actinomycetes introduced into sterile soil with R. japonicum reduced the number of nodules by 35 and 53% when isolate E8 was introduced into the soil, at the time of planting or 28 days before, respectively (Damirgi and Johnson, 1966).

Extracts of certain recently cleared soils, in which subterranean clover failed to nodulate, were frequently toxic to R. trifolii and it was proposed that antibiotic-producing fungi, which proliferate on the organic material left after soil clearing, could lead to nodulation failure (Holland and Parker, 1966).

It has been suggested that bacteriophages which lyse rhizobia, are important in reduction of nitrogen fixation. However, no convincing evidence exists that the ecology of Rhizobium is in any significant way affected by soil-borne bacteriophages.

The fungus Rhizoctonia is reported to cause a 63% decrease in fixed nitrogen per plant in inoculated soybean (Orellana et al, 1976).

Mycorrhiza are reported to improve seed yield and other traits of the nodulating isoline Hardee, but not the non-nodulating isoline (Shenk and Hinson, 1978).

Bdellovibrio is an inhabitant of soil which probably exists in nature as an obligate parasite. Strains feeding on R. meliloti, R. trifolii and cowpea rhizobia have been found in many soils. When these vibrios are provided with cowpea rhizobia in culture, they kill many host cells, so they might be responsible for the death of rhizobia in soil (Alexander, 1975).

Protozoa are suspected as predators of rhizobia in soil. Alexander (1975) reported that R. japonicum introduced into a soil declined in number and that protozoa were the cause. Each protozoa appeared to consume thousands of bacteria, but nevertheless 6.3×10^7 rhizobia/g were left after 12 days. It was further shown that ciliate Tetrahymena eliminated R. japonicum if replication of rhizobia was to be prevented by chloramphenicol.

Tetrahymena feeding on rhizobia in the absence of the inhibitor did not reduce their number below 10^6/ml, but reduced the number of non-replicative rhizobia to 270/ml.

Nematodes were shown to interfere with the rhizobia-legume association. A large proportion of cowpea nodules were occupied in varying degrees, from those showing larval penetration only to conditions where nematodes predominated (Robinson, 1961). Soybeans infected with Heterodera glycines (race 1) showed reduced nodulation and nitrogen fixation, whereas plants infected with other races did not show these symptoms when cyst nematodes were introduced 14 days after rhizobia inoculation (Barker et al, 1972). The greatest inhibition occurred when soybeans were inoculated Simultaneously with R. japonicum and race 1 of the soybean cyst nematode.

Two insects are known to attack legume nodules: Sitona lineata and Rivellia sp. Sitona larvae mature within nodules of a number of legumes, such as Vicia faba, peas and sometimes lupine in Europe. Larvae of Rivellia spp attack nodules of Glycine javanica resulting in 50-70% of the nodules being damaged (Diatloff, 1965).

3.4.13 Amount of Nitrogen Fixed by Different Legumes

Various reports deal with the amounts of nitrogen fixed by legumes. In general, pulses fix nitrogen less than forage legumes; however, this depends largely on the crop itself and on factors interfering with the growth of the crop. See Table 10 (Nutman 1976).

Rizk (1968) studied the amounts of nitrogen fixed by legumes under Egyptian conditions (Table 11). It is clear that clover is in the lead followed by the pulses.

Russell (1961) presented the results of a two year rotation of legume-cereal, the cereal being either rye or barley (Table 12). They show that the yield of the following cereal crop largely depends on the amount of nitrogen the legumes add to the soil. The amount of nitrogen fixed varies from 79 to 505 kg/ha. Leguminous crops grown for seed (peas, field beans, soybeans and groundnuts) show a tendency to reduce the nitrogen content of the soil, whereas legumes grown for their leaf (clovers, sweet clover, and lucerne) increase the nitrogen content.

Rizk (1968) reported that the nitrogen content of the soil varies according to the crop cultivated. As indicated in Table 13, the increase in nitrogen of soil varies between 34 and 259 kg/ha with legumes, but, with non-legumes like barley, sesame and chickoria, the nitrogen content varies between 7 and 38 kg/ha. It is apparent from the table that plants reduce the nitrogen contents in the soil during the growth period.

Inoculation of seeds, and field inoculation with soil from a field where inoculated soybean had previously been grown, both produced 73-94% greater yield compared to the control (Saxena and Tilak, 1975). Without application of nitrogen, following an inoculated soybean crop the yield of wheat was more than 65% higher than when it followed an uninoculed soybean crop. The response was equivalent to about 30 kg N/ha.

A series of field experiments on nitrogen fertilization of paddy transplanted after non-leguminous crops and after berseem showed that paddy transplanted in soils previously cropped with non-legumes was more responsive to added nitrogen than after clover. Yields of rice after non-legumes gradually increased as the rate of nitrogen increased up to 96 kg/ha, whereas the yield of paddy after clover reached its maximum at 24 kg N/ha. (Serry et al, 1970).

Table 10 NITROGEN FIXED BY PULSES, FORAGE, GREEN MANURE AND SHADE TREES
(kg N/ha/yr; Nutman, 1976)

P L A N T	Average	Ranges
A. PULSES		
Vicia faba	210	45-552
Pisum sativum	65	52- 77
Lupinus spp.	176	145-208
Phaseolus aureus (green gram)	202	63-342
Phaseolus aureus (mung)	61	-
Cajanus cajan (pigeon pea)	224	168-280
Vigna sinensis (cowpea)	198	73-354
Canavalia ensiformis	49	-
Cicer arietinum (chickpea)	103	-
Lens culinaris (lentil)	101	88-114
Arachis hypogaea (groundnut)	124	72-124
Cyamopsis tetragonoloba (guar)	130	41-220
Calpogonium mucunoides (calapo)	202	370-450
B. FORAGE, GREEN MANURE, SHADE TREES		
Centrosema pubescens (centro)	259	126-395
Desmodium intortum and D. canum (tick clover)	897	-
Leucaena glauca	277	74-584
Lotononis bainesii	62	-
Sesbania cannabina	542	-
Stylosanthes sp (stylo)	124	-
Mixes of centro and stylo	115	-
Phaseolus atropurpureus (siratro)	291	-
Mikanea cordata	120	-
Pueraria phaseoloides (kudzu)	99	-
Enterolobium saman	150	-

Table 11 NITROGEN FIXED BY DIFFERENT LEGUMES UNDER EGYPTIAN CONDITIONS
(after Rizk, 1968)

C r o p	Stage of growth	Nitrogen fixed kg/ha	g/kg dry wt.
Berseem Fahl	Single cut	67	17
Berseem Miskawy	1st cut	38	
	2nd cut	88	
	3rd cut	62	18
	4th cut	31	
	Seeds	19	
Lupine (termis)	Mature	138	20
Broadbean	Mature	136	17
Fenugreek	Mature	105	23
Chickpea	Mature	98	23
Lentils	Mature	83	22
Groundnut	Mature	79	16
Soybean	Mature	40	14

Note.Original figures in kg/feddan converted to kg/ha to nearest kg.

Table 12 AMOUNT OF NITROGEN FIXED BY LEGUMINOUS CROPS, AND THEIR INFLUENCE ON A FOLLOWING CEREAL CROP
(after Russell, 1961)

	Nitrogen harvested in		Gain or loss of nitrogen in the soil	Total nitrogen fixed by legume	Yield of cereal grain cut
	Leguminous crops	Cereal crops			
	kg/ha	kg/ha	kg/ha	kg/ha	kg/ha
Lucerne	335	74	137	504	26
Clover	140	57	129	291	21.7
Sweet clover	190	57	94	302	21.2
Soybean	197	33	-9	179	13.2
Field beans	115	28	-22	78	11.9
Cereal every year	-	25	-11	-	3.0

Conversion factor used for lb/ac to Kg/ha = 1.12

Table 13 CHANGES IN SOIL NITROGEN CULTIVATED WITH LEGUMES AND NON-LEGUMES
(after Rizk, 1968)

C r o p s	Stage of growth	Change, Kg N/ha
A. LEGUMES		
Broad bean	Immature	- 10
Lentil	Mature	+ 34
Lentil	"	+ 86
Fenugreek	"	+ 166
Termis (lupine)	"	+ 122
Chickpea	"	+ 91
Groundnut	"	+ 82
Soybean	"	+ 58
Berseem fahl	Single cut	+ 101
Berseem miskawy	4 cuts	+ 259
B. NON-LEGUMES		
Barley	Immature	- 26
Barley	Mature	+ 7
Sesame	Mature	+ 38
Chickoria	4 cuts + seeds	+ 22

Note - Conversion factor used for feddan to ha = 2.4 (figures rounded)

i. Transfer of nitrogen to companion plant

There are four possible routes for the transfer of legume-fixed nitrogen from the soil to the companion plant: l.) excretion of nitrogenous compounds from the nodules of the legume to the growing medium, 2) release of nitrogenous material to the soil from droppings of grazing animals, 3) decomposition of aerial parts of legume plants, and 4) the decay of root and nodule tissues.

Whitney (1975) reviewed nitrogen fixation by legume-grass mixtures such as Desmodium intortum, D. canum and Centrosema pubescens with and without pangola (Digitaria decumbens) and napier (Pennisetum purpureum) grasses (Table 14).

Table 14 NITROGEN FIXED BY THREE LEGUMES IN PLASTIC-LINED CINDER PLOTS (Whitney, 1975)

Legume or mixture	Net kg N/ha/yr recovered in tops	roots	total
Desmodium canum alone	45	49	94
D. canum + pangola[1]	35	12	47
D. canum + napier[2]	106	30	136
Centrosema pubescens alone	257	14	271
C. pubescens + pangola	118	14	132
C. pubescens + napier	108	15	123
Desmodium intortum alone	350	56	406
D. intortum + pangola	330	30	360
D. intortum + napier	327	51	378

[1] Digitaria decumbens

[2] Pennisetum purpureum

Net nitrogen-fixation, including secretion of nitrogen in the root mass, was low for D. canum except for the mixture with napier grass. Centrosema pubescens was intermediate in result, but nitrogen-fixation with grass was also low. D. intortum was effective in fixing nitrogen (supplying about 400 kg N/ha/year in pure stand and only slightly less in the grass-legume mixture).

In another study (Table 15) nitrogen fixed and transferred between Desmodium and pangola grass was determined. D. canum fixed nearly 100 kg N/ha/year, of which an estimated one third was transferred into the pangola grass. Although D. intortum fixed 264 kg N/ha/year, it transferred only slightly more nitrogen to the grassland than did D. canum, amounting to one eighth of the total nitrogen fixed. D. Intortum in pure stand fixed the most nitrogen.

Table 15 NITROGEN CONTRIBUTION BY TWO DESMODIUM FORAGE LEGUMES IN ASSOCIATION WITH PANGOLA GRASS. (after Whitney, 1975)

Treatment	Apparent kg N/ha/yr		
	Legume %	fixed	transferred
D. canum (uninoc.) + pangola[1]	26	80	26
D. canum (inoc.) + pangola[1]	27	97	32
D. intortum (90 cm rows) + pangola[1]	54	213	42
D. intortum (45 cm rows) + pangola	64	264	37
D. intortum (45 cm rows) alone	100	290	-

[1] Digitaria decumbens

ii. Legumes as green manure crops

The economic feasibility of using legumes as green manure crops, as a general procedure in tropical agriculture remains to be proven. In contrast to temperate conditions, decomposition of the plant residues is too fast, and the residual effect is generally short-lived (and not much beyond one year). However, there are other beneficial short-term effects such as improved water infiltration and water holding capacity, and control of plant diseases and pests. Green manuring of maize with Crotalaria juncea, Canavalia ensiformis, or Stizolobium deeringianum corresponded to the addition of 60 kg N/ha/yr of fertilizer N, but the economics of this did not compare favourably with adding higher levels of mineral nitrogen fertilizer. Table 16 shows the superiority of Crotalaria juncea over other legumes and sorghum as a green manure for field beans, Phaseolus vulgaris, (Dobereiner and Campelo, 1975).

Table 16 EFFECT OF GREEN MANURE INCORPORATED IMMEDIATELY BEFORE PLANTING ON THE YIELD OF FIELD BEANS (PHASEOLUS VULGARIS) IN SAO PAULO STATE, BRAZIL. (Dobereiner and Campelo, 1976)

Green manure	Fresh weight incorporated	Grain yield kg/ha
Crotalaria juncea	57.797	800
Cajanus cajan	25.026	637
Lablab purpureus	31.656	591
Tephrosia candida	13.884	609
Sorghum vulgare	56.025	416
None		418
L.S.D.		165

4. BLUE-GREEN ALGAE

4.1 INTRODUCTION

Recognition of blue-green algae as nitrogen fixers dates back to Frank in 1889 when he noticed that soils containing blue-green algae fixed nitrogen. Prantil also, reported (in 1889) that impure cultures of Nostoc fixed nitrogen and other workers around the same period observed similar phenomena. But this was followed by a period of doubt until Drews in 1928 published evidence that Anabaena variabilis, Anabaena sp, and Nostoc punctiforme grew well in pure culture in a medium free of combined nitrogen (Stewart, 1975).

4.2 NITROGEN-FIXING BLUE-GREEN ALGAE

With the developments of techniques such as ^{15}N and acetylene reduction assay, it became evident that there are three main groups of blue-green algae: unicellular, filamentous heterocystous and filamentous non-heterocystous, that include types which fix nitrogen (Stewart, 1977, Tables 17 and 18). In all these groups, the nitrogenase appears remarkably similar and the main differences appear to be in the localization of the enzyme and in the ways in which it is protected from inactivation by oxygen (Fig. 11).

4.3 CLASSIFICATION

According to the 8th edition of Bergey's Manual of Determinative Bacteriology, blue-green algae are classified in Division I which includes the phototrophic procaryotes, "The cyanobacteria". The division includes 3 classes:

Class I : Blue-green photobacteria
Class II : Red photobacteria
Class III: Green photobacteria.

(Class I and II represent part I and include the phototrophic bacteria).

All phototrophic procaryotic organisms use water as an electron donor and hence produce oxygen in the light.

The characteristics of cyanobacteria are summarized by Stanier (1974) as follows:

> Cells are always enclosed by a rigid, multilayered wall with an inner peptidoglycan layer. The wall may in turn be surrounded by a gelatinous or fibrous sheath. Most cyanobacteria are mobile at some stage of development; motility is always of the gliding type, dependent on surface contact. The cytoplasmic region is traversed by an extensive system of paired photosynthetic lamellae (thylakoids); the outer surfaces bear characteristic granules (phycobilisomes) composed of aggregates of the phycobiliprotein pigments.
>
> Some cyanobacteria are unicellular, others consist of colonies of cells (filaments), either simple or branched. Reproduction of unicellular forms may occur by binary fission, by multiple fission or by the aerial release of apical cells (exospores) from a sessile individual. Forms that consist of filaments grow by repeated intercallary cell divisions and reproduce either by random fragmentation of the filament or by terminal release of short motile chains of cells (hormogonia). Certain filament formers can produce specialized cells known as akinetes and heterocysts. Akinetes are

Table 17 HETEROCYSTOUS BLUE-GREEN ALGAE REPORTED TO FIX NITROGEN IN PURE CULTURES (Stewart, 1977)

Anabaena ambigua
A. azollae
A. cycadeae
A. cylindrica
A. fertilissima
A. flos-aquae
A. gelatinosa
A. humicola
A. levanderii
A. naviculoides
A. variabilis

Anabaena sp.

Anabaenopsis circularis
Anabaenopsis sp.

Aulosira fertilissima

Calothrix brevissima
C. elenkinii
C. parietina
C. scopulorum

Chlorogloea fritschii

Cylindrospermum gorakhpurense
C. licheniforme
C. majus
C. sphaerica

Fischerella major
F. muscicola

Hapalosiphon fontinalis

Mastigocladus laminosus

Nostoc calcicola
N. commune
N. cycadeae
N. entophytum
N. muscorum
N. paludosum
N. punctiforme
N. sphaericum

Scytonema arcangelii
S. hofmanni

Stigonema dendroideum

Tolypothrix tenuis

Westiellopsis prolifica

Table 18 NON-HETEROCYSTOUS ALGAE REPORTED TO FIX NITROGEN IN PURE CULTURES (Stewart, 1977)

Gloeocapsa 795
Gloeocapsa 6501
Lyngbya 6409
Oscillatoria 6407
Oscillatoria 6412
Oscillatoria 6506
Oscillatoria 6602

Phormidium sp.
Plectonema boryanum
Plectonema 6306
Plectonema 6402
Rhaphidiopsis indica
Trichodesmium (impure)

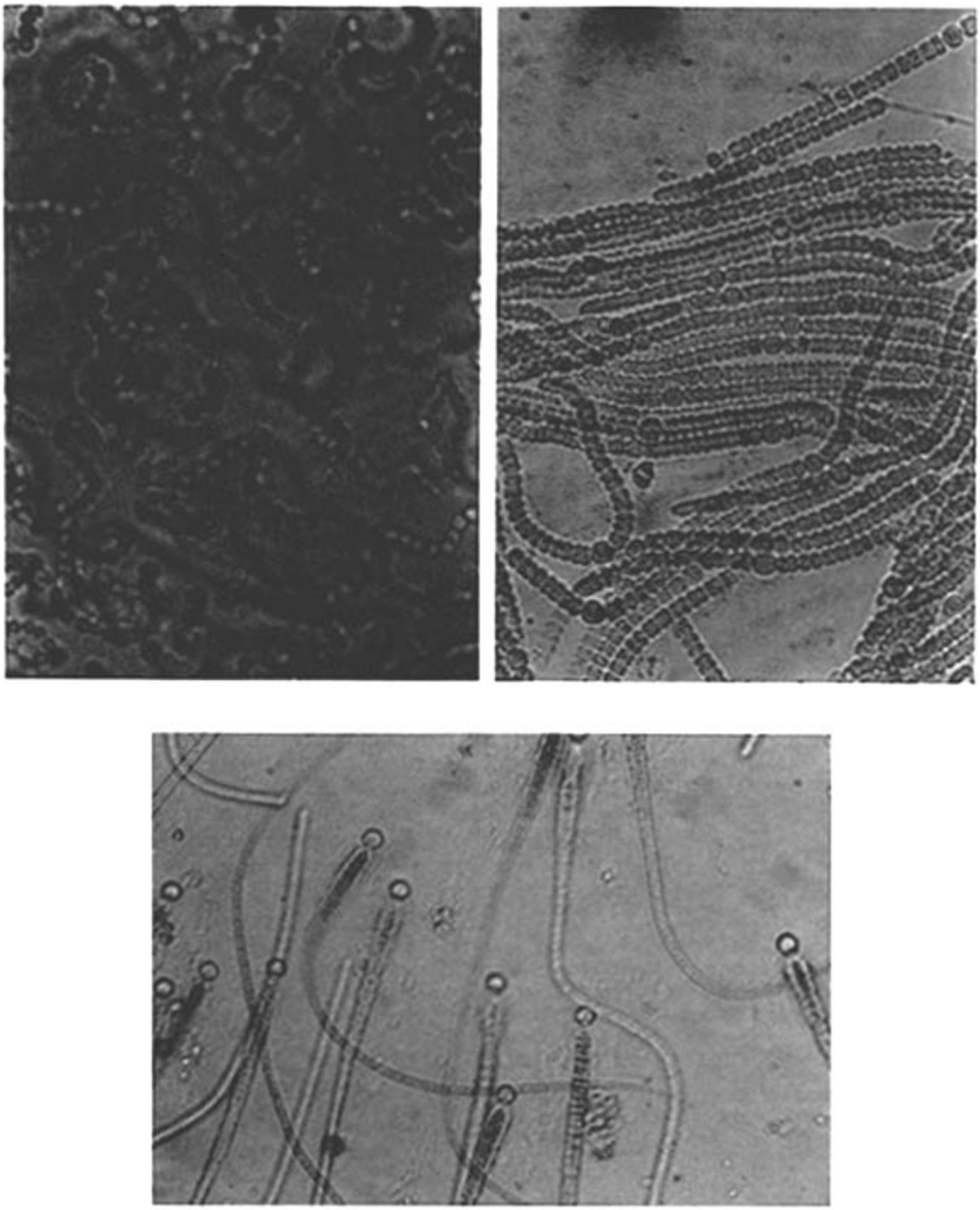


Fig. 11 Some nitrogen-fixing blue-green algae.
From top, *Gloeotrichia* sp., *Nostoc* sp. and *Anabaena* sp.
(contributed by I. Watanabe)

larger than the vegetative cells in the filament and represent a resting stage, which germinates with a release of a hormogonium. Heterocysts are non-reproductive cells, distinguishable from the adjoining vegetative cells by the presence of refractile polar granules and of a thick outer wall: they are believed to be physiologically specialized cells that serve as sites of nitrogen-fixation.

The characteristic photopigments include chlorophyll as the only Chlorophyllous pigment and phycobiliproteins (allophycocyanin, phycocyanic and sometimes phycoerithrin). The cellular absorption spectrum has a peak at approximately 680 nm attributable to chlorophyll(a) and a broad absorption band with one or more peaks, attributable to phycobiliproteins between 560 and 630 nm.

A detailed discussion on the status of the classical taxonomy of blue-green algae is given by Desikachary (1973).

4.4 GENETICS OF BLUE-GREEN ALGAE

Stewart (1975) indicated that little progress had been made in knowledge of the genetics of nitrogen-fixation by blue-green algae. A few reports have dealt with the isolation of large numbers of mutants and some eveidence has been presented of genetic recombination, and of transformation.

4.5 THE SITE OF THE NITROGENASE ENZYME IN BLUE-GREEN ALGAE

Nitrogen-fixing blue-green algae have three distinct cell types: vegetative cells, spores (akinetes) and heterocysts. The cellular localization of nitrogenase in heterocystous algae has been, and still is to some extent the subject of much debate. Although in theory, heterocysts, akinetes and vegetative cells could all produce a nitrogenase, the akinetes have been eliminated because tests have shown that they do not reduce acetylene (Fay, 1969).

Experimental evidence has been presented that heterocysts do not have photosystem II and hence would not be able to assimilate CO_2. It has also been shown that carbon supplied as $^{14}CO_2$ is fixed in the vegetative cells and transferred into heterocysts. The transport takes place intrafilamentously, possibly via microplasmadesmata which transverse the membrane separating the cells. Nitrogen fixed in the heterocysts is also thought to travel intra-filamentously (Stewart et al, 1969).

The discoveries of aerobic nitrogen-fixation in two strains of the unicellular algae Gloecapsa alpicola and in unialgal cultures of the non-heterocystous alga Trichodesmium sp, and the anaerobic nitrogen-fixation of the non-heterocystous filamentous alga Plectonema boryanum 594, suggest that vegetative cells of heterocystous algae might at least fix nitrogen under anaerobic conditions even if they cannot do so aerobically.

4.6 ISOLATION AND PURIFICATION OF BLUE-GREEN ALGAE

Under natural conditions, algae grow as mixed communities which include species and genera. It is therefore obvious that in order to study individual species, the organisms must be cultivated without other types or forms present

4.6.1 Unialgal Cultures

The term “unialgal culture” refers to one containing only one species of alga in the presence of other organisms.

Felfoldy and Zssusza (1959) used the serial dilution pour-plate technique on agar plates containing inorganic salts after shaking to separate individual cells. De (1939) obtained unialgal cultures by distributing the algal cells over the surface of the solidified agar with the aid of a sprayer, bent glass rod, and brush or platinum loop. Gerloff et al (1950) used a micromanipulator as a means to isolate the desired algae.

4.6.2 Bacteria-free Cultures of Algae

As the term indicates, the culture should contain a single species of alga, and be free from other bacteria.

Phototactic motion (creeping movement) is useful as a means of eliminating the bacteria. During multiplication, the algae spread over the sterile agar surface and the attaching bacteria are left behind.

Shalan (1974) used silica gel plates to obtain bacteria-free Anabaena naviculoides and A. variabilis. Wieringa (1968) obtained bacteria-free cultures of Anabaena variabilis, A. azolla, Nostoc muscorum, N. paludosum, N. piscinale, Nodularia sp and Microchaete sp by using heat treatment.

Pure cultures of Nostoc linckia, N. muscorum and Anabaena variabilis were obtained with the aid of a gamma cell producing 6.48×10^4 rad/h at the irradiation site (Kraus, 1966). These pure cultures were obtained upon using 288 000 rad for 4.75 h.

Gerloff et al(1950) employed ultraviolet irradiation to obtain bacteria-free cultures of Aphanizomenon flos-aquae and Diplocystis aeruginosa by exposing the algal suspension in the hormogonial state to ultraviolet light from a quartz-mercury vapour lamp for 20-30 minutes. Pure cultures were obtained of Hapalosiphon fontinalis and Anabaena variabilis by UV-irradiation for 15-20 minutes at a distance of 40 cm, but Calothrix elenkinii only required UV-irradiation for 1-3 minutes at a distance of 12 cm (Taha, 1963). The antibiotics streptomycin (0.05-0.4 mg/ml) and penicillin G (4-40 IU/ml) were used to obtain pure cultures of Scenodesmus acuminatus and S. quadsicanda, Navicula sp and Euglena sp after a few subcultures had been made.

Gupta et al (1956) succeeded in obtaining pure cultures of algae using mercuric chloride. Chlorine water, 25 ppm or 2.5% for 2 minutes, was used to purify cultures of Anabaena cylindrica and A. variabilis. An electric field of 500 V and 1.0 mA for 48 hr gave bacteria-free cultures of Nostoc (Coler et al, 1969), and Shalan (1974) secured the best results using silica gel and sodium tellurite treatment.

4.6.3 Tests for Purity of Algal Cultures

Two sources of errors may occur when purifying algae:

a. the contaminating bacteria may not be able to grow in the medium used;
   or
b. some bacteria may be embedded in the mucilage of the alga and, failing to pass out into the medium, will not produce visible contamination.

In order to ascertain the purity of algal cultures they must be tested on a set of culture media suitable for various possibly contaminating bacteria:

i. caseinate agar;
ii. soil extract sucrose nitrate broth and agar;
iii. albumate agar;
iv. litmus milk:
v. dextrose peptone broth (1% each of dextrose and difco proteose peptone);
vi. bacto-tryptophane broth (difco, 1% solution);
vii. 2% mannitol in tap water + 0.02% potassium monohydrogen phosphate (a medium suitable for Azotobacter).

4.6.4 Methods for Testing the Ability of Blue-Green Algae to fix Nitrogen

In experiments designed to show the nitrogen fixation by a given alga, two points should be carefully considered:

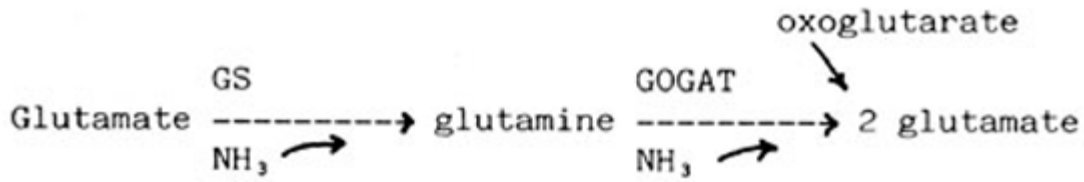
i. the alga must be in absolutely pure culture, because even with a contaminant which is known to be otherwise incapable of fixing nitrogen, it is possible that it may fix nitrogen in the presence of the organism under examination;
ii. there must be no doubt whatsoever that any increase in fixed nitrogen in a culture, is due to the uptake of free nitrogen.

It is important to make sure that nitrogen is not absorbed in the form of ammonia or oxides of nitrogen from the atmosphere. Before being passed over the culture, air must be purified from traces of combined nitrogen by passing it through a solution of sodium bicarbonate and a 25% solution of sulphuric acid; the wash bottles should be the type filled with glass bubbles.

Some methods which have been developed for testing include the following

- ^{15}N Tracer. Williams and Burris (1952) developed a method for testing Calothrix parietina (Appendix D.l);
- Microkjeldahl (Appendix A.2);
- Acetylene reduction (Appendix D.3);
- Detection of ammonia. Stewart and Rowell (1975) indicated that it is possible to get the blue-green alga Anabaena cylindrica to secrete ammonia into the medium. This method depends on two facts.

1) The main pathway of incorporation of newly fixed nitrogen by nitrogen-fixing organisms involves the enzyme glutamine synthetase and also (usually)the glutamate synthase.



2) The analogous methionine sulphoximine (MSO) inhibits the GS pathway of ammonia incorporation and when this happens, the newly fixed nitrogen, rather than being incorporated into general cell metabolism is excreted into the medium and can be measured calorimetrically.

MSO should be used at the rate of 1 N mol(use filter-sterilized M50, e.g. 1 ml of 100 μ mol solution in 99 ml of the cultures). MSO experiments should not exceed 12 to 24 hours. If left for 48 hours or more the experiment may cease to function.

Extracellular ammonia can be detected by the method adopted by Solorzano (1969) (Appendix D.2).

4.7 GROWTH RATE OF BLUE-GREEN ALGAE

The specific growth rate of blue-green algae depends on many factors such as the nature of the organism, quality and quantity of light available, carbon dioxide concentration, nutrient status, metabolites, etc. (Venkataraman, 1969).

Hoogenhout and Amesz (1965) tabulated the growth rates of photosynthetic organisms. The number of doublings per day for some blue-green algae are presented in Table 19.

Table 19 NUMBER OF DOUBLINGS PER DAY OF SOME BLUE-GREEN ALGAE (Hoogenhout and Amesz, 1965)

Name of algae	K	Temp.°C	Illumination	Medium
Agmenellum quadriplaticum	8.00	39.0	?	S
Anabaena cylindrica	0.96	25.0	I	S
A. variabilis	3.90	34.5	I	S
Anacystis nidulans	11.50	41.0	I	S
Cylindrospermum sphaerica	0.17	25.0	nI	S
Gloeotrichia echimula	0.20	26-27	nI	S
Microcystis aeruginosa	1.60	23.0	?	S
Nostoc muscorum	3.10	25.0	?	S
Nostoc muscorum	2.90	32.5	I	S
Schizothrix calcicola	3.40	30.0	I	S
Synechococcus lividus	8.50	52.0	?	S
Synechococcus sp	9.90	45.0	?	nS
Synechocystis sp	8.00	37.0	?	S
Tolypothrix tenuis	8.00	38.0	I	S

I: illuminated at saturation
nI: non-saturating light intensities
?: unknown
S: synthetic medium
nS: non-synthetic medium
K: number of doublings

4.8 ECOLOGY AND FACTORS AFFECTING NITROGEN-FIXING BLUE-GREEN ALGAE

4.8.1 Distribution

Blue-green algae occur in many habitats extending from the Antarctic to the Tropics, but they are only in certain localized areas such as rice fields, mesotrophic and eutrophic fresh waters and alkaline hot spring regions, and in symbiosis with other plants. Blooms of Trichodesmium occur in some tropical waters.

4.8.2 Nutrients for Blue-Green Algae

i. Carbon

Blue-green algae have a photosynthetic system which enables them to utilize carbon dioxide as the sole carbon source in the light.

Carbon dioxide bubbled into the culture of the algae at a
Rate of 0.5 to 5% of air passing through increases the growth rate.
However, higher levels of carbon dioxide frequently result in a drastic decrease in pH even in a buffered medium.
Aeration with air containing 3% carbon dioxide favours the growth of Nostoc spongiaeforme. While 5% carbon dioxide in air is toxic to cultures of Anabaena cylindrica,it is satisfactory for very dense cultures of this species at saturating light intensity.

It appears that glucose, sucrose and mannitol stimulate the growth of, and nitrogen-fixation by Tolypothrix tenuis, Nostoc muscorum and Calothrix brevissima under continuous illumination, while sodium acetate or citrate have no effect or are inhibitory (El-Sayed, 1974).Shalan (1974) observed that in the presence of light, maximum growth and nitrogen-fixation by Anabaena sp and Aulosira sp took place with molasses, followed by glucose and mannitol at a concentration of 0.2%.

ii. Nitrogen

Nitrogen-fixing blue-green algae can readily assimilate inorganic and organic nitrogen, and when combined nitrogen is available it may be assimilated prerentially thus inhibiting fixation. This inhibitory effect depends however on the level and type of combined nitrogen, the exposure periiod to it, the physiological status of the algae and whether or not the experiments are carried out with whole cells or extracts.

iii. Phosphorous

De and Sulaiman (1950) reported that the addition of phosphates to rice fields, whether in soluble form as potassium phosphate or in insoluble form as calcium phosphate, stimulated nitrogen-fixation by blue-green algae. Similarly,De and Mandal (1956) showed that superphosphate at the rate of 67 kg/ha of P_2O_5 incerased nitrogen-fixation only in soils relatively poor in this nutrient.

El-Nawawy et al (1972) demonstrated that the
nitrogen fixed by the)bdueogreentad that the growth and/or nitrogen fixed by the blue-green alga T. tenuis were incresed with an increase in the P_2O_5 level up to 63 ppm, but were depressed with 123 ppm. Similarly, dry weight and/or nitrogen fixed by N. linckia var. arvense were increased and reached a maximum at the level of 40 ppm P_2O_5, after which a gradual decrease occurred (Taha et al,1974).

iv. Calcium

Calcium has been shown to be required for the growth of blue-green algae. It plays a particularly important role in the maintenance of plasma membrane, salt formation with colloids and cell wall materials. It has been shown that calcium is required for Anabaena cylindrica whether the alga grows with molecular or combined nitrogen and at least 20 ppm of calcium are needed for optimum growth and this element cannot be replaced by strontium (Allen and Arnon,1955).

v. Trace elements

Cobalt is an essential element for nitrogen-fixation by Nostoc muscorum and Calothrix sp although the requirement is very small, (0.004 ppm is sufficient for growth). Molybdenum is essential for the growth of seven species of blue-green algae in medium without combined nitrogen. It has been shown that the optimum concentration required for Anabaena cylindrica in a nitrate medium is 0.1 ppm, whereas more than 0.2 ppm is required to give optimum growth in elemental nitrogen. The optimal concentration of molybdenum for T. tenuis in the absence of combined nitrogen or in the presence of N0 -N was found to be 0.01 ppm, but when ammonium sulphate, urea^{3} or monosodium glutamate were supplied no growth response to molybdenum was observed. Nostoc commune responded to added molybdenum reaching a maximum at about 4×10^{3} mg/l after which a decline in algal growth occurred (Taha and El-Rafai, 1962).

Boron has been found essential for maximum growth of various blue-green algae. Nostoc commune G when inoculated into a boron-free medium cannot develop naturally and requires boron for good growth. Cell counts in boron-free cultures show relatively small numbers; chlorosis starts slowly during the 3rd week and by the end of the 8th week the alga becomes comnpletely white.

Manganese is needed as a trace element in the medium for good growth of some blue-green algae. The quantities required differ depending upon the type of the alga and the environmental conditions under which it grows. It has been shown that the presence of manganese in the medium stimulates intensely both nitrogen-fixation and photosynthesis in the culture of Anabaena ossillaroides and Hapalosiphon fontinalis f.globosus.

4.8.3 Light

Stewart (1975) has cited numerous studies on the relationship between light and nitrogenase, both with free-living blue-green algae and with lichens. Generally there is a correlation between nitrogen-fixation and acetylene reduction in natural ecosystems. The relationship is also complicated by other factors such as the effect of desiccation, while in aquatic systems light intensities may be excessive at the water surface. For example the activity of samples collected at 0.9m depth in Lake Mendota was found to be more than that in the surface waters (Rusness and Burris, 1970).

Under controlled laboratory conditions it is still debateable as to whether algae grow better when given a dark period each day or whether maximum growth per unit of time is obtained when the cells are continuously illuminated. Allen and Arnon (1955) showed that the rate of growth of Anabaena cylindrica was essentially the same when the alga was grown under 11 hours or 13 hours of darkness or 24 hours of light each day. However, Ukai et al (1958) found a light intensity of 8 000 lux and a temperature of 32°C suitable for optimum steady growth of Tolypothrix tenuis without causing any sign of bleaching. Half saturation of growth was observed at light intensities of 1 000 and 1 500 and 2 500 lux at 26, 32 and 38°C, respectively. They also showed that the apparent half-saturation of nitrogen-fixation was obtained at an intensity lower than the lowest examined i.e. 800 lux.

In the case of the algae Hapalosiphon fontinalis, Anabaena variabilis and Calothrix elenkinii it was found that they did not grow in the dark in a nitrogen-deficient medium even in the presence of various alcohols, sugars and organic acids. The best algal growth occurs under continuous illumination and the nitrogen-fixing capacity increases with increasing light intensity (Fogg and Than-Tun, 1960).

Granhall (1970) reported that the reduction of acetylene by the blue-green alga Nostoc punctiforme isolated from Swedish soils, was at light intensities below 100 lux and the highest saturation level for the process was about 3 000 lux.

4.8.4 Temperature

Blue-green algae are able to withstand temperature extremes. Nitrogen-fixation by hot spring algae was noted in Alaska and in Yellowstone National Park where the upper limit of temperature was near to 60°C. Blue-green algae dominant at 40-60°C include species of Mastigocladus (Stewart, 1975). In hot springs in Japan, potential nitrogen-fixing species of Anabaena Calothrix Cylindrospermum, Hapalosiphon, Mastigocladus, Nostoc and Scytonema all occur at temperatures ranging from 30-50°C.

At the low temperature extreme, in the Antarctic, blue-green algae are abundant, and the most common genera in this habitat are Nostoc, Scytonema hofmanni, Anacystis and Coccochloris.

4.8.5 pH

Blue-green algae are found in most abundance in natural or slightly alkaline habitats, and rarely in acid ones.

Several workers have determined the growth of nitrogen-fixing blue-green algae as affected by hydrogen ion concentration. Nostoc muscorum G can grow in a medium containing nitrate at pH 6.9-9.0. pH values of 7.0-7.5 were found to be optimum for Anabaena flos-aquae and A. scherematrevi; pH 8.0 for Aphanizomenon and pH 9.0 for Microcystis aeruginosa. HapaZosiphon fontinalis, Anabaena variabilis and Calothrix elenkinii grew at pH 7.0-8.0. Maximum growth of Nostoc linckia var.arvense was attained at pH 8.0

4.8.6 Desiccation

Desiccation is one of the more important factors regulating the nitrogenase activity of blue-green algae. Nitrogen-fixation by Nostoc and Collema in Antarctica is greatly affected by the snow-free seasons and desiccation.

In the hot deserts of America, blue-green algae occur particularly in any puddled areas, in intermittent streams and transitory pools, in lichen symbiosis and under pebbles, stones and rocks. In temperate regions, in situ rates of nitrogen-fixation by Nostoc in sand -d une ecosystems were found to be much higher in areas shaded by long grass than in more open, desiccated areas (Stewart, 1965).

4.8.7 Oxidation Reduction Potential

Blue-green algae grow well under reducing conditions at a redox of 170 to 600 mV. Singh (1961) reported that in Indian paddy soils the redox potential of the soil stabilises on the reducing side at the time of puddling, and then thick growths of blue-green algae develope.

4.8.8. Pesticides

Pesticides commonly used in agriculture may or may not affect the growth of blue-green algae, depending on the type, concentration and time of application of the compound. For example it has been found that Gamma BHC stimulates algal population in rice fields by inhibiting the predators which feed on algae.

On the other hand Hamdi et al (1970) evaluated the effect of the herbicides molinate, propanil, trifluralin and 2,4-D. They found that dry weight and nitrogen contents of the alga T. tenuis were reduced in the presence of these when added either at the beginning or 10 days after the start of growth (Table 20). Chlorophyll synthesis was stimulated by low levels of molinate, trifluralin and 2,4-D in both treatments but was inhibited by propanil.

Venkataraman and Rajyalakshimir (1971,1972) also examined the effect of various pesticides. They found that while most of the strains of Anabaena could tolerate 100 ppm of “Ceresan M” a few were sensitive to a concentration as low as 0.1 ppm. T. tenuis and Aulosira fertilissima tolerated high concentrations of “Ceresan M”, “Zineb”, 2,4-D and “Dalapon”. However, A. fertilissima was found to be sensitive to substituted urea compounds such as fluometaron, diuron and linuron, as well as the triazine herbicide propazine.

Table 20 EFFECT OF HERBICIDES ON DRY WEIGHT, NITROGEN AND CHLOROPHYLL CONTENT OF T. TENUIS (Hamdi et al, 1970)

Herbicides (ppm)	After inoculation on 10 day-old culture					
	Dry wt. (g)	Nitrogen (mg)	Chloro-phyll (mg)	Dry wt. (g)	Nitrogen (mg)	Chloro-phyll (mg)
Molinate						
0.25	280	8.3	3.2	395	10.6	3.2
2.50	290	7.8	2.0	371	6.7	3.2
25.00	101	6.6	1.0	340	8.2	2.9
Trifluralin						
0.25	307	8.5	3.3	221	10.3	2.7
2.50	243	7.7	2.1	289	9.2	3.5
25.00	96	8.6	0.6	309	7.9	2.5
2,4-D						
0.25	316	10.4	8.6	276	9.5	2.8
2.50	375	9.5	2.7	280	8.3	2.9
25.00	375	9.2	3.1	270	9.1	2.5
Propanil						
0.18	308	9.2	3.3	407	10.4	3.8
1.80	231	4.6	1.0	427	6.4	2.8
18.00		No growth		199	1.8	1.1
Control	448	13.0		448	13.0	2.0

4.8.9 Algal Pathogens

Blue-green algae are attacked by various organisms such as aphids, protozoa, fungi, bacteria and viruses (Singh, 1978) and these manifest themselves as pale or white spots, or rings, on algal lawns. The variability in algal population from field to field and the sudden disappearance of algae are generally attributable to attack by these organisms. The chytrids and bacteria (Myxobacteria, Cytophaga) are found in many habitats and laboratory work has shown that they can infect algal lawns to plague proportions. Daff, Begg and Stewart (1970) isolated a virus pathogenic to Plectonema, Phormidium and Lyngbya from a variety of fresh waters in Scotland. Blue-green algal viruses from rice fields in India have been studied in detail (Singh and Singh, 1967).

4.8.10 Salinity

Blue-green algae are widely distributed in marine ecosystems, although they are seldom dominant. Hof and Fahmy in 1933 (Fogg et al, 1973) divided the algae into two physiological groups: halotolerant and halophilic. For example a halotolerant species is Calothrix scopulorum,while an example of a halophilic species is Spirulina subsalo.

El-Nawawy et al (1968) reported that the fixation of atmospheric nitrogen by the local strain of blue-green alga, Calothrix sp was slightly affected by 1 747.5 ppm of sodium chloride, and severely depressed by 3 395.5 ppm. However, concentrations of 795 and 1 590 ppm had no influence on the nitrogen-fixation of the alga. Growth of the alga was not affected appreciably until the highest concentration of 4 191.0 ppm was used.

Khadr (1975) showed that growth of Nostoc calcicola increased as a result of increasing the sodium chloride concentration up to 9 000 ppm after which it began to decline. Growth of Anabaena variabilis was slightly increased by 9000 ppm of sodium chloride but it was markedly decreased by 12 000 and 15 000 ppm. Higher concentrations were found to be lethal for this alga. Growth of Anabaena oryzae and Nostoc muscorum significantly decreased at 21 000 ppm and the same concentration of sodium chloride was lethal to Anabaena naviculoides.

Brock (1976) isolated the halophilic blue-green alga Aphanothece halophytica from the Great Salt Lake in Utah,U.S.A.. The optimum salinity for growth of this organism was found to be about 16% of sodium chloride, but it could grow very slowly even in a saturated (30%) solution.

4.9 AMOUNTS OF NITROGEN FIXED BY BLUE-GREEN ALGAE

4.9.1 Under Laboratory Conditions

Amounts of nitrogen fixed under laboratory conditions vary according to the species, type of media, period of incubation, method of determination, etc. Table 21 summarizes some of the available data on the amounts of nitrogen fixed in culture media.

4.9.2 Under Field Conditions

Various reports indicate that there is an increase in the nitrogen content of the soil through algal inoculation. Studies using ^{15}N tracer methods showed that algae fixed about 22.5 kg of nitrogen per hectare and that the crop absorbed 4.8 kg nitrogen (Nishigaki et al, 1953).

Table 21 AMOUNTS OF NITROGEN FIXED BY DIFFERENT BLUE-GREEN ALGAE IN LIQUID CULTURES

Species	Nitrogen fixed			
	Period days	Quantity	Reference	
Anabaena naviculoides	60	3.6 mg/100 ml	De	1939
A. oryzae	60	4.4 "	"	"
A. variabilis	60	5.7 "	"	"
Nostoc commune	60	70.6 mg/1	El Borollosy	1972
Tolypothrix tenuis	60	141.9 "	"	"
A.naviculoides	45	154.4 "	El Sayed	1978
A. oryzae	45	270.2 "	"	"
N. calcicola	45	187.3 "	"	"
N. muscorum	45	175.5 "	"	"
A. naviculoides	55	83.0 "	Khadr	1975
A. oryzae	55	117.0 "	"	"
A. variabilis	55	92.7 "	"	"
N. calcicola	55	99.3 "	"	"
N. muscorum	55	101.3 "	"	"
N. sphaericum	55	83.0 "	"	"
A. ambigua	45	5.7 mg/100 ml	Singh	1942
A. fertilissima	45	6.7 "	"	"
Aulosira fertilissima	45	8.7 "	"	"
Cylindrospermum gorakhpurense	45	5.0 "	"	"
N. amplissimum	90	1.26-1.45 g/l	Subramanyan and Sahay	
N. sphaericum	90	1.2 -1.3 "	"	1964
T. campylonemoides	90	1.6 -1.81 "	"	"
Westiella sp.	90	1.0 -1.1 "	"	"
A. variabilis		27 mg/100 ml	Taha	1963
Calothrix elenkinii		57 "	"	"
Hapalosiphon fontinalis		22 "	"	"
Cylindrospermum sp.	60	78.6 mg/1	Taha et al	1973
N. linckia var arvense	60	91 "	"	"
N. paludosum	60	61.6 "	"	"
N. piscinale	60	87 "	"	"
Anabaenopsis sp.	60	34 mg/100 ml	Watanabe et al	1951
Calothrix brevissima	60	52 "	"	"
Nostoc sp.	60	31 "	"	"
T. tenuis	60	96 "	"	"

Singh (1961) attributed a fixation of about 80 kg of nitrogen per hectare in maize fields in Utter Pradesh to algae. Sankaram (1971) showed a gradual increase in the organic matter content due to algal inoculation, although the increase was small (Table 22).

Table 22 BUILD UP OF ORGANIC MATTER DUE TO ALGAL INOCULATION (Sankaram, 1971)

	Increase/decrease of organic matter over uninoculated fields	
	1 9 6 4	1 9 6 5
Control	0.06	0.09
F Y M (20 kg N/ha)	0.02	0.08
Green manure (20 kg N/ha)	0.05	0.09
Ammonium sulphate (20 kg N/ha)	0.06	0.04
Urea (20 kg N/ha)	0.08	0.01

In West Bengal, De and Mandal (1956) estimated a fixation rate of 15-49 kg N/ha over a six week period. When the soils were fertilized with phosphorus, the amount of nitrogen fixed was calculated to be 18-69 kg N/ha during the same period. Watenabe et al, (1951) reported nitrogen-fixation by Tolypothrix tenuis inoculated in rice fields in Japan to be about 22 kg N/ha.

Granhall (1976) reported data on in situ nitrogen-fixation rates in Swedish soils near Uppsala by a 0-2 cm layer of blue-green algae. The results varied between 0.06 and 27.7 mg $N/m^2/hr$ in the light. The main nitrogen fixing genera were Anabaena, Cylind.rospermum and Nostoc. Clay soils were among the most suitable habitats for blue-green algae and the environmental factors, e.g. moisture, temperature, pH and light conditions, were nearly optimum during incubation. Heterotrophic fixation by bacteria, as judged from samples kept in the dark for 24 hr, was sometimes observed and varied between 0.09 and 1.2 mg $N/m^2/hr$. Henriksson et al(1972) reported values from 0.01 to 4.5 mg $N/m^2/hr$ for aerobic algal fixation in other clay soils near Uppsala. Stewart (1967) reported values of 2.5 mg $N/m^2/yr$ for alga in sand dunes.

4.10 TRANSFER OF FIXED NITROGEN

Blue-green algae may liberate large quantities of ammonia extracellularly under certain conditions. Many reports also indicate that a variety of organic compounds are liberated (Stewart, 1975).

Studies on symbiotic systems have shown clearly that the nitrogen fixed by blue-green algae is rapidly transferred to the host. Bergersen et al (1965) have shown that nitrogen fixed in cycads appeared in all parts of the plants within 48 hours. Nitrogen fixed in lichens was transferred to the mycosymbiont within 3 hours. ^{15}N appeared in the angiosperm in a Gunnera system within 1.5 hours of supplying ^{15}N to the alga.

Pot and field experiments have shown that the nitrogen fixed by blue-green algae becomes available to associated rice plants, which results in increased crop yield.

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4.11 PRODUCTION OF VITAMINS AND GROWTH SUBSTANCES BY BLUE-GREEN ALGAE

Many algae produce vitamin B_{12} and this process is promoted by the addition of cobalt salt but is not much affected by carbon or nitrogen sources (Venkataraman et al, 1964; Venkataraman and Neelakanthan, 1967).

In addition to vitamins, the algae produce auxin-like growth substances; and considerable amounts of ascorbic acid (Vaidya et al, 1970). The algae liberate these substances either by excretion or autolysis into the surrounding medium, from which the crop plants can assimilate them.

4.12 MASS CULTIVATION OF BLUE-GREEN ALGAE

An extensive review of the knowledge on the engineering, biological and operational aspects has been presented by Venkataraman (1969). The following are summaries of the techniques used by various workers for the production of blue-green algae biomass.

4.12.1 Japanese Technology (Watenabe et al, 1959)

i. Preliminary shake culture

The alga from agar slants was transferred to round flasks which were constantly shaken at 32°C. The cultures were illuminated by incandescent lamps set in the inner wall of the incubator. Two week old cultures were used as inocula for the tank cultures.

ii. Tank culture

The tank was a 30 litre pyrex glass cylinder housed in a water bath thermostatically controlled at 32°C. The water bath was provided with three windows for illuminating the culture from outside. The light intensity was controlled by adjusting the position of the light source. The tank had a stirrer and a pipeline for aeration. The carbon dioxide enriched air (3%) was bubbled at a rate of 350 ml/min into the culture solution. A rubber tipped wiper was also provided to detach the alga from the walls of the glass cylinder. Prior to inoculation, the chamber and the culture medium were sterilized with steam for 15 min at 0.1 kg/mm^2, When the culture attained a density of about 2 g/1 (dry wt) about half the culture was withdrawn and used as the inoculum for outdoor cultures. The tank was then filled with fresh medium to the initial volume. The average growth rate obtained with this tank culture was 0.2 g dry wt/1/day.

iii. Closed circulation system

For outdoor culture, the culture chamber was made of a long flat polyvinyl bag of 300 litre capacity, the two ends of which were connected with each other through a 3 cm diameter and 8 m long tube. The chamber was directly connected to the culture tank to facilitate aseptic transfers from it to the outdoor growth chamber. The culture was constantly stirred by a whirl pump at a rate of 20 1/min and 5% CO_2 enriched air was bubbled into the culture. The culture was cooled by pouring tap water over the culture bag and the intensity of incident light was controlled by a bamboo blind. Before starting the culture, the entire system was sterilized with 1% hydrogen peroxide solution which was later removed by steaming in sterilized water. The maximum yield obtained with this outdoor device was 7.9 g dry wt/m^2/day.

The culture media used for the three types of culture described above are presented in Table 23. For the tank and outdoor cultures tap water was used instead of distilled water.

Table 23 CULTURE MEDIA USED TO PROPAGATE BLUE-GREEN ALGAE (Watanabe et al, 1959). [1]

Culture media	Pre-culture (g/l)	Tank culture (g/l)	Outdoor culture (g/l)
K_2HPO_4	0.3	0.3	0.3
$Mg\ SO_4.7H_2O$	0.2	0.2	0.2
$CaCl_2$	0.05	0.05	0.05
$FeCl_3$	trace	trace	
KNO_3		0.1	0.1
$(NH_4)_2\ MoO_4$		0.02	
Na_2CO_3			0.2
EDTA			0.1
A_5-micronutrients			1.0 ml

[1] See Appendix D.8 for composition.

iv. Gravel culture

A special volcanic gravel was used commercially known as Kenuna-Tsuchi, for growing Tolypothrix tenuis. Sieved gravel was washed several times with distilled water, soaked in nitrogen-free medium and steam-sterilzed at 1.1 kg/cm^2 for 15 min. The gravel was then mixed with concentrated algal suspension and the resulting inoculated gravel was placed in polyvinyl tubes under weak illumination. After about 4 weeks incubation the gravel cultures were transferred to vinyl bags or glass bottles and kept at room temperature. These were used for field inoculation.

4.12.2 Indian Technology

Venkataraman (1961) considered the rate and cost of algal production and developed simple techniques such as dry sand, foam and open air soil cultures as described below.

i. Dry sand culture

Sieved quartz sand is washed well, boiled in distilled water for about 2 hours and dried at 120°C. The dried sand is soaked in nitrogen-free medium and steam-sterilized at 1.4 kg/cm^2 for 30 min. The sand is then mixed with a concentrated algal suspension and gradually sun dried; When dried, it is preserved in tubes or paper bags and stored. However, an obvious advantage of sand culture is that the sand particles, being heavy, sink into the mud, thus hampering the rapid growth of adhering alga.

ii. Foam culture

Synthetic sponge is cut into rectangular cubes of 2-4 cm, boiled in water, soaked in nitrogen-free medium under suction and sterilized. The sponge pieces are then spread on metallic boats, layered with vinyl sheeting. The top of the boat is covered by a thick vinyl sheet, supported by a number of arch-shaped metallic wires attached to the side of the boat. The sponge pieces are either mixed with algal suspension before spreading them on the boats or the algal suspension is spread over them. The boats carrying the inoculated sponge pieces are incubated under weak illumination. Within 3 to 4 days, the sponge pieces become completely covered with the algal growth, and after a suitable period of incubation, they are removed and stored in plastic bags. When these algal-covered sponge pieces are inoculated into waterlogged soils, they float on the surface of the water and the algae begin to grow rapidly and spread over the surface of the water.

iii. Open air soil cultures

The merit of this system lies in the possibility it offers for farmers to prepare their own inoculum. The basic principle is to grow the algae using natural sunlight, under conditions simulating a rice field. A thin layer of soil (2.5 cm) is spread in a rectangular tray of galvanized iron measuring 0.6 x 0.9 m and flooded with 2.5 cm of standing water (Fig. 12). After the soil settles down, the specific strain of alga is inoculated into these trays with phosphate (0.2 g Na_2HPO_4/1) and molybdenum (0.2 mg MoO_3/1). The trays are kept in the open air and within 4 to 5 days, the entire surface of the water is covered by a copious growth of the inoculated alga. The standing water is allowed to dry and the dried algal mat cracks and peels off as flakes. This dried material is stored in plastic bags and used for field inoculation.

An average production of about 20 g/m^2/day (dry wt) can be continuously obtained in this way, with a ten day collection schedule. The limitations of this method are essentially climatic, for during the winter months, the rate of production slows down and during rainy seasons the production may have to be temporarily suspended. However, the rate of production during clear warm days and the inexpensive operational costs outweigh some of these limitations. Such algal nursery beds may be laid out in a farmer's field, and enclosed by earth embankments. Useful strains may be inoculated into these beds just before the rainy season, or where water is available, the beds may be kept waterlogged.

Prior to seeding, the beds may be given a dressing of lime, phosphate and molybdenum to promote the growth of the inoculum. Even insoluble phosphate, like rock phosphate, can be used. The algal scum (or mat) can be scooped out, dried and used for broadcasting over the main field. It may also be possible to direct the inflow of irrigation water over the beds, so that the algal material may be distributed over the entire field.

iv. Sewage propagation of blue-green algae

Venkataraman (1972) suggested the possibility of producing blue-green algae in sewage oxidation ponds. The bloom of Oscillatoria grown in such ponds at Ahmedabad was found to have a high manurial value for rice-plants.

Fig. 12 Algal cultivation in trays containing soil (contributed by P.K. Singh)

4.12.3 Chinese Technology

The practice of algalization in China was observed on a FAO field tour in 1977. At the Agricultural Research Station, Nanjing, an inoculum mixture of Anabaena and Nostoc spp is prepared in flasks containing suitable media under sterile conditions. The algae are then transferred into larger glass bowls to grow under non-sterile conditions.

The fresh algae are subsequently used to inoculate a nursery plot (5-7 m long, 1 m wide and 20 cm deep) at the rate of 150 g/m^2. The water in the nursery is 6-7 cm deep. After about 7 days, the algae in the plot attain a density of 500-1 000 g/m^2. The nursery is covered with transparent plastic sheets to protect the plants from cold.

After preparing the main field for rice cultivation, it is flooded and 750 kg/ha of algae are spread as inoculum. The algae grow and attain 7.5 t/ha and even up to 16 t/ha within 10-15 days at temperatures above 30°C.

4.12.4 Other Methods

Other methods have been developed to produce mass cultures of blue-green algae for different research purposes, e.g. enzymology.

i Bath cultures

Haystead et al (1970) grew A. Cylindrica at 25°C in 10 litre batches in aspirators illuminated.with four 40 watt “grolux” tubes at a distance of 10 cm from the vessel. The culture was agitated with a magnetic stirrer and sparged with the required gas mix at a rate of 200 1/h. The culture medium used was the nitrogen-free one of Allen and Arnon (1955).

ii Continuous culture of blue-green algae

Bone (1971) grew Anabaena flos-aquae A-37 in continuous culture. The medium used had the following composition per litre:

Na_2CO_3 - 25 mg; K_2CO_3 - 25 mg; Tris buffer - 100 mg; $MgSO_4.7H_2O$ - 75 mg; $CaCl_2.6H_2O$ - 60 mg; $FeSO_4.7H_2O$ - 60 mg; Na EDTA - 120 mg;

H_3BO_3 - 2.86 g; $MnSO_4.4H_2O$ - 1.81 g; $ZnSO_4.7H_2O$ - 0.222 g; $Na_2MoO_4.2H_2O$ - 0.391 g; $CuSO_4.5H_2O$ - 79 mg; $CaCl_2.6H_2O$ - 39 mg;

Phosphate, the limiting nutrient, was added as K_2HPO_4 and nitrate as KNO_3.

iii. Mass culture for various micro-algae

Soeder (1976) reviewed the technical production of micro-algae, other than nitrogen-fixing blue-green algae, e.g. Chlorella and Scenodesmus (green-algae) and Spirulina. Open air ponds for the cultivation of micro-algae are either circular ponds or horizontal channels with mechanical or hydropneumatic generation of turbulence by inclined planes. Further experimentation is needed to refine these methods before they can be generally adopted for the production of blue-green algae.

4.13 ALGALIZATION OR THE APPLICATION OF BLUE-GREEN ALGAE TO SOIL

4.13.1 Methods of Inoculating Blue-Green Algae

Singh (1961) broadcasted the algal stratum mixed with 1 kg of lime onto paddy fields after the rice had been transplanted. Sankaram (1971) mixed an algal suspension with washed sand and spread the mixture over the field one week after transplanting rice.

Venkataraman (1972) mixed multistrain soil cultures in a bucket of water containing molybdenum (0.5 kg sodium molybdate/ha) and sprinkled it uniformly over the water in the rice field, one week after transplanting the rice.

The multistrain inoculant contained T. tenuis, Cylindrospermum muscicola, Aulosira fertilissima and Nostoc sp. The idea of using a mixture of algae was to offset the ecological or edaphic changes to any one particular strain in a given locality. All these four forms have been found to have good competitive ability under field conditions. The only shortcoming is that when several forms are used as inocula, the ultimate proportion of individual strains in the soils tends to be unpredictable under field conditions; this variation is not too important, because the establishment of any efficient form will suffice.

When direct sowing of rice seeds by broadcasting is adopted, seed inoculation may be achieved as follows: the suspension of the soil culture is mixed rapidly with the rice seeds and 2-3 kg of calcium carbonate per 10-20 kg of seed, until all seeds are evenly coated. The coated seeds are then air dried in shade and sown.

As regards the optimum quantity of inoculum (1-6 kg/ha) and frequency and time of application, the results are rather inconclusive. Of the different methods of inoculation, soil application and seed inoculation seem to be preferable (Venkataraman, 1972)

4.13.2 Algae on Soil Effect of Blue-Green and Rice Yield

In India many investigators have studied the response of rice to algal inoculation. De and Sulaiman (1950) ran a 5 year pot culture experiment to find out the effect of algal growth in soils on the yield of rice. In this experiment, rice plants were grown in pots, some of which were kept in the dark (without algae), while in others the soil surface and supernatant water above were exposed to light (with algae present). The results showed that during the first, second and third years, the yields with algae present and absent were not very different; However, rice yields were much higher in the fourth and fifth years. Upon determination of nitrogen at the end of the experiment there was a considerable increase in nitrogen in the soils in which algae grew abundantly, but in the soils in which algae were absent, a loss of nitrogen was evident.

Singh (1961) indicated that after inoculation of rice (variety T9) with Aulosira fertilissima there was an increase in yields of 368% in pots and 114.8% under field conditions over the controls (Table 24).

Table 24 EFFECT OF INOCULATION OF *AULOSIRA FERTILISSIMA* ON AVERAGE GROWTH AND YIELD OF A VARIETY OF PADDY (T9) (After Singh, 1961)

Experiment	Height of plants cm	Tillers per plant No.	Earheads per plant No.	Paddy per plot 2	Increase over control %
Control	73	3	3.5	5.0	-
Pots (inoculated)	141	15	12.7	23.4	368.0
Fields (inoculated)	133	12	10.3	-	114.8

Date of inoculation: 23.7.1955. Date of harvest: 3.12.1955.

In a field experiment, Subrahmanyan et al (1965) found that inoculation with blue-green algae increased the yield of rice over control by 30%. An increase of 266% was obtained in pot experiments by inoculation of A. fertilissima (Sundara Rao et al, 1963).

Relwani and Subrahmanyan (1963) observed that partial soil sterilization and blue-green algae with a nutrient mixture consisting of lime (1 000 kg/ha) superphosphate (100 kg/ha) and molybdenum (0.28 kg sodium molybdate/ha) contributed to an increase in yield of 45% under field conditions. This was comparable with the yield obtained by the application of 20kg nitrogen/ha as ammonium sulphate.

Jha et al (1965) reporting on field experiments in Sabour indicated that algal inoculation increased yield by 40% and its effect in combination with phosphate was similar to that of green manure plus phosphate (Table 25).

Table 25 EFFECT OF *T. TENUIS* ON GRAIN YIELD (kg/ha) OF 498-2 A "AMAN" VARIETY OF RICE. (Jha *et al*, 1965)

	1962-63	1963-64	1965-66
1. Control	2 191	1 826	2 899
2. Algal inoculation	3 012	2 490	3 686
3. Phosphate (45 kg P_2O_5/ha)	2 265	1 827	3 810
4. (2) + (3)	3 186	2 859	3 810
C.D. 5%	719.2	664.2	577

Venkataraman and Goyal (1969) showed that combined application of algae and nitrogen at all levels was better than the corresponding nitrogen level alone and was comparable to the next higher level of nitrogen (Table 26).

Table 26 GRAIN YIELD OF PADDY (IR 8) DUE TO ALGAL INOCULATION AT DIFFERENT LEVELS OF NITROGEN [1] (Venkataraman and Goyal, 1969)

Treatments	1967 Grain yield (g)	1968 Grain yield (g)
l. Control	3.96	2.16
2. Algae	5.83	4.40
3. 20 kg N/ha	6.90	4.45
4. 40 kg N/ha	10.31	7.68
5. 60 kg N/ha	13.55	11.58
6. 80 kg N/ha	14.40	14.20
7. 100 kg N/ha	13.86	14.88
8. (3) + algae	7.30	7.26
9. (4) + algae	10.58	9.26
10. (5) + algae	16.15	13.40
11. (6) + algae	18.03	14.55
12. (7) + algae	24.88	19.55
C.D. at 5%	3.66	1.94

[1] Pot culture experiment values are average of six replications.

In a trial conducted in a farmer's field with rice variety IRB, it was observed that algal inoculation increased the yield by 20% over control with no chemical nitrogen and by 12% over 100 kg nitrogen/ha alone (Venkataramar and Goyal, 1968). In another cultivator's field with no added nitrogen fertilizer, the same authors observed an increase of about 18% in the grain yield of ASD 5 rice variety compared with an application of a fertilizer mixture of superphosphate and sodium molybdate (Table 27).

Table 27 GRAIN YIELD OF RICE VARIETY ASD 5 DUE TO ALGAL INOCULATION (Venkataraman and Goyal, 1968)

Treatments	Grain yield kg/h	increase %
l. Control	5 859.4	
2. Fertilizer mixture (112.1 kg superphosphate + 0.25 kg Na molybdate/h)	6 453.4	10.13
3. Fertilizer mixture + algae (1 kg dry wt/h)	7 470.7	27.48 over 1 18.76 over 2

Sankaram (1971) indicated (in a cultivator's field in Bihar) that algal inoculation gave a comparable yield from rice variety BR 34 to that obtained with an application of 45 kg N/ha (Table 28).

Table 28 EFFECT OF *T. TENUIS* ON THE GRAIN YIELD (kg/ha) OF RICE VARIETY BR 34 AT SABOUR IN BIHAR (Sankaram, 1971)

	1964-1965	1965-1966
1. Control	2 464	2 196
2. Phosphate (45 kg P_2O_5/ha) + algae	3 491	3 174
3. 45 kg N/ha + 45 kg P_2O_5/ha	4 064	3 514
C.D. 5%	588	438

Recent investigations have shown that, irrespective of varietal differences among the high yielding fertilizer-responsive dwarf rices, all respond to algal inoculation (10-15% increase in the yield) even in the presence of high levels of nitrogen fertilizer (100-150 kg/ha). This results in an increased output per unit input of added fertilizer (3-5 kg grain/kg N) (Venkataraman, 1972).

In Japan, large-scale tests of the effect of Tolypothrix tenuis on rice yield were started by Watanabe in 1951 at eleven experimental farms and continued for four years (Watanabe, 1956). As a result of applying this blue-green alga, the yield of rice (averaged over 11 fields) was increased by 2.7% in the first year and 8.4% in the second year. In the first year, only one third of the alga multiplying in the rice field decomposed and was absorbed by the rice plants; the rest remained as nitrogen fertilizer in the soil. In one of the experiments, it was clear that the effect of inoculating the rice field with this alga was almost similar to that of manuring with 71.68 kg/ha ammonium sulphate. Finally it was shown that 4 years after inoculation with the alga, fields of rice could yield 128% more than uninoculated controls.

In the U.S.S.R., an increase in crop yield of 13 to 20% has been attributed to the application of Amorphonostoc punctiforme. An accumulation of from 3 to 15 mg/100 g of soil was observed after inoculation of sodpodzolic soil with blue-green algae (Shtina et al, 1968).

In China, Ley (1959) reported that inoculation with Anabaena azotica increased rice yield by about 24% under field conditions. During a field trip in China, (FAO 1977), it was observed that the practice of inoculating rice with a mixture of Anabaena sp and Nostoc sp caused an increase of 10% in rice yield.

In Egypt, El-Nawawy et al (1958) inoculated one gram (dry weight) of T. tenuis into lysimeters (20 x 2 m) planted with rice to give a final concentration of 494 g/ha. This was found to be sufficient to satisfy the nitrogen requirements of the rice. Abou-el-Fadl et al (1964) studied the response of rice to inoculation with T. tenuis together with ammonium sulphate, compost, straw and superphosphate. In general, inoculation with T. tenuis significantly increased soil nitrogen but had no effect on rice yield. Apparently the addition of organic matter (compost or straw) stimulated nitrogen-fixation but the ammonium sulphate was inhibitory. Furthermore the addition of superphosphate together with ammonium sulphate or compost markedly inhibited nitrogen-fixation.

Ibrahim et al (1971) also studied the effect on rice yield and soil nitrogen of adding T. tenuis, phosphorus and nitrogen fertilizer. Addition of the alga increased the grain, straw and nitrogen yield, as shown in Table 29. Algal inoculation, without fertilizer, increased the yield of rice grain and straw by 4.2% and 19.3% respectively. In the presence of phosphorus, the increase was 7.0% and 56.6% with maximum yield being obtained from alga plus 0.5 g phosphorus per pot.

El-Borollosy (1972) evaluated the response of rice to inoculation with the following blue-green algae: N. linckia var. arvense, Cylindrospermum sp, N. paludosum, N. piscinale, T. tenuis and N. commune (Fig. 13). Five rice seeds were planted in washed sand in pot cultures. These pots were divided into two series. The first was irrigated with nitrogen-free nutrient solution, while the second was treated in the same manner but supplemented with nitrogen. The highest total nitrogen levels in the plants and in the sand cultures, as well as the highest fresh and dry weight of rice plants after 115 days were observed inpots inoculated with T. tenuis, followed by the local isolate N. linckia var. arvense, then in decreasing order: N. piscinale, Cylindrospermum sp, N. commune and N. paludosum. The results are well illustrated in Fig. 13.

Table 29 EFFECT OF INOCULATION WITH ALGA T. TENUIS ON THE YIELD OF RICE (g/pot) (Ibrahim et al, 1971)

Treatment	Grain			Straw		
	-alga	+alga	% increase	-alga	+alga	%increase
Control	10.45	10.89	4.2	20.42	24.37	19.3
0 g N+P[1]	12.37	13.23	7.0	21.06	32.99	56.6
0.25 N+P	24.14	28.03	16.1	32.35	52.96	63.7
1.0 N+P	36.44	39.20	7.5	41.98	62.77	39.5
2.0 N+P	39.15	41.48	5.9	45.68	63.63	39.3
L.S.D. 5%		1.97			14.63	

[1] The content of P is stable at 0.5.

Field experiments on the inoculation of rice paddies by T. tenuis were carried out over two successive years (Abou-el-Fadl et al, 1967). The results (Table 30) showed that the average rice yield following beans was much higher than that planted after wheat. When no phosphate was added, the application of alga, or nitrogen, at the two levels gave approximately similar results. With calcium superphosphate, the effect of 240 g alga was generally less than that caused by 24 kg N, but the difference was insignificant when rice followed beans. Moreover, the effect of 480 g alga together with phosphorus was much smaller than without phosphorus.

Abou-el-Fadl, et al (1970) studied the response of rice to algal inoculation as affected by crop rotation and the time of application of nitrogen and phosphorus fertilizers. The data obtained (Table 31) indicate that: i) in the first four experiments, the best rice yield was from fertilization with 48 kg N and 36 kg P_2O_5 per ha. Algal inoculation gave a yield similar to fertilization with 24-48 kg N/ha. The algae + 36 kg P_2O_5 did not give a better yield than that without P_2O_5; ii) with the application of superphosphate two weeks before and ammonium sulphate four weeks after transplanting, algal inoculation caused an increase of 23.1% in yield (experiment 5); iii) in experiment 6 where 72 kg N/ha was added four weeks after transplanting, algal inoculation increased rice yield by 22.3%; iv) when the amount of ammonium sulphate was reduced to two thirds the recommended dose (experiments 7 and 8), inoculation with alga gave a greater yield than when maximum levels of ammonium sulphate alone were used. In general, the greenhouse and field experiments indicated that blue-green algae served as a useful nitrogen source for rice growth.

In a pot experiment Khadr (1975) showed that the nitrogen contents of rice plants increased as a result of algal inoculation (Table 32).

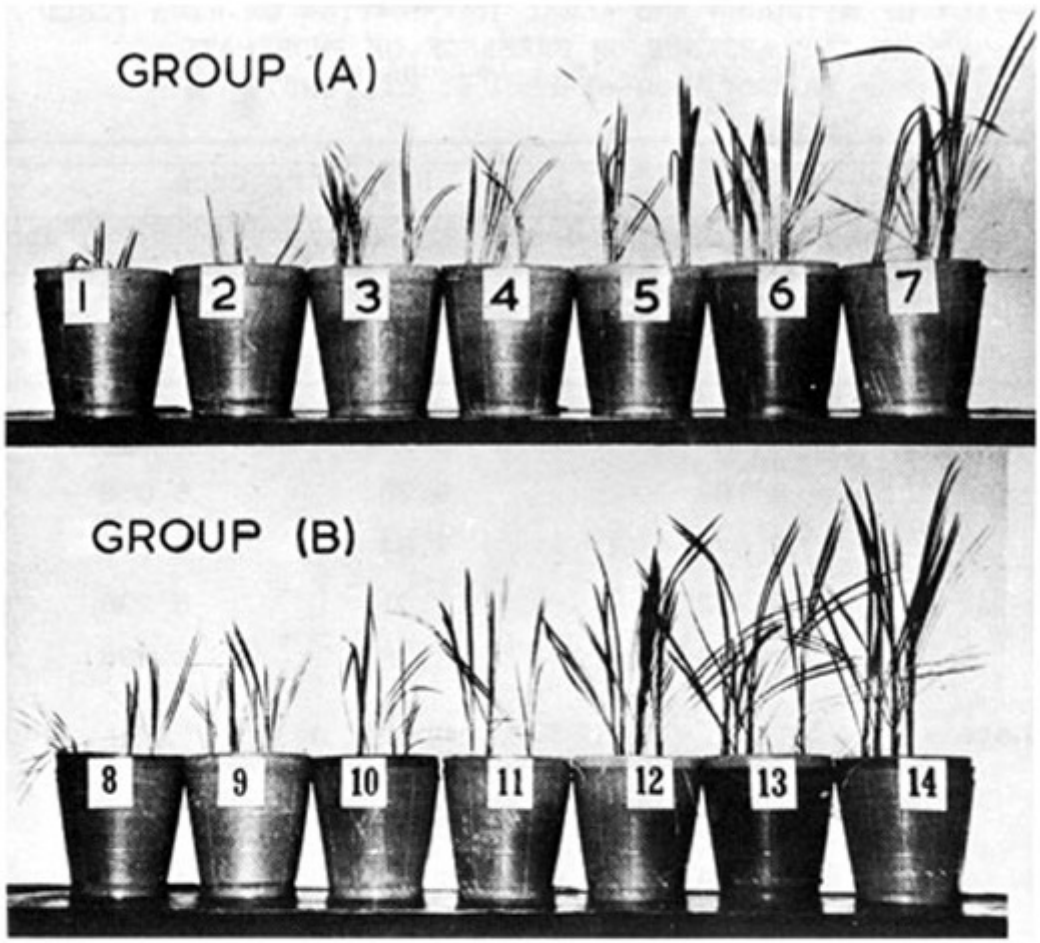


Fig. 13 Effect of soil inoculation with blue-green algae on growth of rice plants

Group A irrigated with N-deficient solution.
Group B irrigated with N-supplemented solution.

1, 8 not inoculated
2, 9 inoculated with *N. paludosum*
3, 10 inoculated with *N. commune*
4, 11 " " *Cylindrospermum* sp
5, 12 " " *N. piscinale*
6, 13 " " *N. linckia* var. *arvense*
7, 14 " " *T. tenuis*

(contributed by M.A. El-Borrolosy)

Table 30 EFFECT OF NITROGEN AND ALGAL INOCULATION ON RICE YIELD IN THE ABSENCE OR PRESENCE OF PHOSPHATE
(After Abou-el-Fadl et al, 1967)

Treatment per ha	Preceding crop: Horse bean Yield kg/ha	Horse bean Increase %	Wheat Yield kg/ha	Wheat Increase %
Without Ca-superphosphate				
24 kg N	8 182	9.28	6 058	28.18
48 kg N	8 074	7.84	6 177	30.69
240 g alga	8 312	11.01	6 296	33.21
480 g alga	7 768	3.75	6 476	37.01
With Ca-superphosphate (36 kg P_2O_5/ha)				
24 kg N	8 910	18.99	7 020	48.51
48 kg N	9 108	21.64	6 627	40.21
240 g alga	8 539	14.04	6 130	29.70
480 g alga	7 196	-	6 004	25.20

For consistency, the original figures presented in ardebs and feddans have been converted to metric units. The following conversion factors have been used:

1 ha = 2.4 feddan
1 ardeb = 150 kg (approx)

Field trials in Egypt (Hamissa, Khadr and Gad, 1978) using the blue-green algae A. oryzae (local isolate) and A. fertilissima (imported culture), with and without nitrogen fertilizer, showed that the yield of rice (seed and straw) was significantly increased: 31.6% by the alga A. oryzae. Nitrogen uptake by plants was also increased in the presence of the blue-green alga A. oryzae by 25 to 42.5%. A. fertilissima, however, did not stimulate the response of rice as much as the nitrogen uptake. Algalization combined with 36 kg N/ha caused an increase in yield of 72%; nitrogen fertilizer gave a 45.62% increase.

In another study, Khadr et al (1978) showed that T.Tenuis increased the yield of rice and nitrogen uptake when it was grown in three different Egyptian soils. The addition of nitrogen (36 kg/ha) and phosphorus (36 kg P_2O_5/ha) increased the response to algalization. (Table 33).

Table 31 EFFECT OF CERTAIN FERTILIZERS AND THE PRECEDING CROPS ON THE YIELD OF RICE
(After Abou-el-Fadl *et al*, 1970)

Experimental application			Preceding crops relative yield							
	Time (weeks)		Horse bean	Barley	Clover	Flax	Wheat	Bean	Clover	Wheat
Constituents per ha	N	P								
1) No fertilizer	-	-	100	100	100	100	-	-	-	-
2) 24 kg N	2*	-	109.3	128.2	-	-	-	-	-	-
3) 48 kg N	2	-	107.3	130.2	119	113.4	-	-	-	-
4) 48 kg N + 36 kg P_2O_5	2	2*	121.6	140.2	136.0	129.6	-	-	-	-
5) as (4)	2	2**	-	-	-	-	100	-	-	-
6) 240 g alga	-	-	11.1	133.2	120.5	115.3	-	-	-	-
7) 240 g alga + 36 kg P_2O_5	-	2	114.1	129.7	-	-	-	-	-	-
8) 240 g alga + 48 kg N	2	-	-	-	-	-	-	107.2	-	-
9) 240 g alga + 48 kg N +	2	-	-	-	-	-	-	-	-	-
36 kg P_2O_5	-	2	-	-	-	127.8	-	-	-	-
10) as (9)	4	2**	-	6	-	-	123.1	-	-	-
11) 240 g alga+ 72 kg N	4	-	-	-	-	-	-	122.3	-	-
12) 72 kg N	2	-	-	-	-	-	-	100	100	-
13) as (11) 36 kg + P_2O_5	-	-	-	-	-	-	-	-	-	100
14) 240 g alga+ 48 kg N	-	-	-	-	-	-	-	-	-	-
+ 36 kg P_2O_5	2	2**	-	-	-	-	-	-	-	107.2

* after transplanting

** before transplanting

For consistency, the original figures presented in kg/feddan have been converted to kg/ha.

Table 32 EFFECT OF INOCULATION WITH ALGAE AND NITROGEN FERTILIZATION ON NITROGEN CONTENTS OF RICE PLANTS IN DIFFERENT SOILS (mg N/pot) (Khadr, 1975)

Treatments	Undeteriorated soil		Saline soil		Alkaline soil		Mean of treatment	
	N-content	increase %	N-content	increase %	N-content	increase %	N-content	increase %
Control	53.44	-	53.02	-	39.82	-	48.76	-
+ N	85.92	60.77	69.46	31.01	83.78	110.39	79.72	67.39
A. naviculoides	61.10	14.33	58.08	9.47	47.56	19.43	55.58	14.41
A. naviculoides + N	109.12	104.19	86.86	63.62	91.00	126.02	95.66	97.94
N. calcicola + N	61.00	13.95	61.02	15.09	52.26	31.24	58.23	20.09
N. calcicola + N	101.40	89.72	90.06	69.31	73.96	85.73	88.47	68.26
A. oryzae	59.64	11.60	58.72	10.66	52.50	12.68	56.95	11.65
A. oryzae + N	102.72	92.22	80.90	52.58	82.34	106.78	88.65	83.86
mixed culture	88.14	64.93	65.16	24.78	60.28	51.38	71.19	47.03
mixed culture + N	123.86	131.77	72.66	37.04	60.00	50.68	85.51	73.16
mean of soil	84.63	58.36	69.60	30.96	64.38	61.70		
L.S.D.	0.05		0.01					
Soils	5.05		7.34					
Treatments	9.31		13.53					
Interaction	16.03		23.16					

Table 33 YIELD RESPONSE (AVERAGE OF THREE SOILS) TO ALGALIZATION WITH *T. TENUIS* AND NITROGEN AND PHOSPHORUS FERTILIZERS (Khadr, *et al*, 1978)

	Yield		Nitrogen uptake	
	g	increase %	mg	increase %
Control	16.50	-	181.94	-
Alga	22.85	38.48	245.49	34.92
N* + alga	31.74	92.36	321.10	76.49
P* + alga	31.99	93.87	361.53	98.70
L.S.D. 1%	6.66	-	64.26	-
5%	3.90	-	54.28	-

* N and P were applied at a rate of 36 kg/ha (15 kg/feddan)

4.13.3 Effect of Blue-Green Algae on Crops other than Rice

In addition to the positive effect of algae on rice yield, algalization has a beneficial effect on other crops as well. For example Venkataraman (1972) reported that barley benefited, and the introduction of Azotobacter and algae together increased the yield of oats by 34% (a greater increase than that obtainable by each constituent separately).

Aiyer et al (1972) reported a significant increase in the vitamin C content of tomato fruits through algalization. Singh (1961) reported an increase of 202.7% in grass yield after soil inoculation with Cylindrospermum licheniforme, but no significant effect on the yield of wheat from algal inoculation was observed.

Dadhich et al (1969) observed a significant increase in yields and nitrogen uptake by Capsicum annuum (Chili) and Lactuca sativa (lettuce) due to inoculation with blue-green algae Calothrix anomala. In these cases, a combined application of a nitrogenous fertilizer like urea, and alga, was found to be more effective than an individual application of either urea or alga. This result suggested that the beneficial effect of the alga was largely due to the production of growth promoting substances.

5. FREE-LIVING NITROGEN-FIXING BACTERIA

5.1 AZOTOBACTERIACEAE. TAXONOMY

According to Becking (1974a) the family Azotobacteriaceae comprises four genera: Azotobacter, Azomonas, Beijerinckia and Derxia. Every genus has one or several species as follows:

Genus AZOTOBACTER has four species:

A. chroococcum A. beijerinckii A. vinelandii A. paspali

Genus AZOMONAS has three species:

A. agilis A. insigne A. macrocytogenese

Genus BEIJERINCKIA has four species:

B. indica B. mobilis B. fluminensis B. derxii

Genus DERXIA has one species:

D. gummosa

Normally these organisms fix at least 10 mg of atmospheric nitrogen per gram of carbohydrate consumed, but some Beijerinckia strains fix less (6-9 mg) because of copious polysaccharide production. The efficiency of nitrogen fixation increases appreciably at lower carbohydrate levels or lower oxygen tension. Organic growth factors are not required for growth but for nitrogen-fixation. Trace elements, in particular molybdenum (a specific catalyst of nitrogen-fixation) are required.

5.2 GENUS AZOTOBACTER

The genus Azotobacter is one of the oldest known nitrogen fixers. It comprises four species: A. chroococcum (Fig. 14), A. beijerinckii, A..vinelandii and A. paspali. Azotobacter chroococcum inoculant is known as Azotobacterin.

Fairly recently it has been discovered that A. paspali contributes to the nitrogen resources of Paspalum notatum.

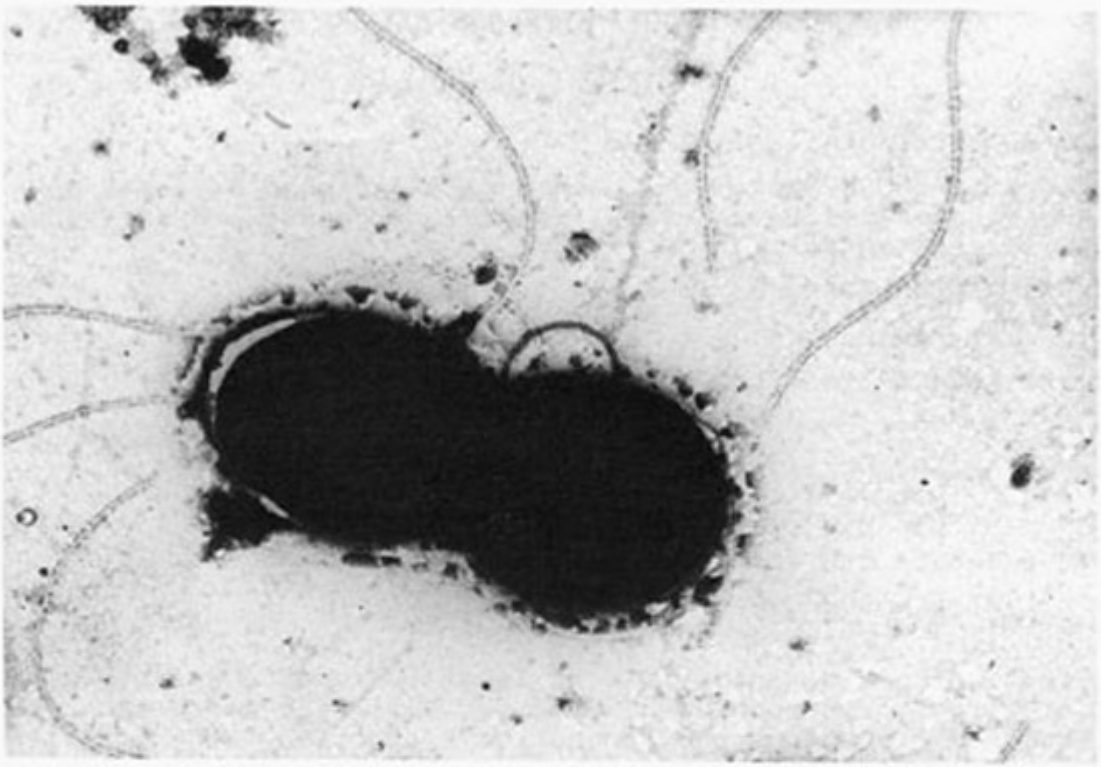
5.3 AZOTOBACTERIN

5.3.1 Historical Background

In 1902 Gerlach and Vogel were the first to study the effect of Azotobacter in soil, on the growth of several plants. They noted no increase in the dry weight of oats, but an increase of 42% in dry weight and 35% in nitrogen content of white mustard and a decrease of 83% and 78% in dry weight and nitrogen content respectively of carrot.

In the Sugar Industry Experimental Station in Prague, Stocklasa (1910) showed that azotobacter caused a 10% increase in the yield of sugar-beet roots, and 35% in the yield of beet tops. With oats, the grain yield was increased by 13% and straw by 16%. Potato was also increased by 31%.

These results, and others reviewed by Rubenchick (1960) led to the production of an azotobacter preparation in 1937 in all Soviet Union Research Institutes. It was called azotogen, but the name was later changed to azotobacterin.



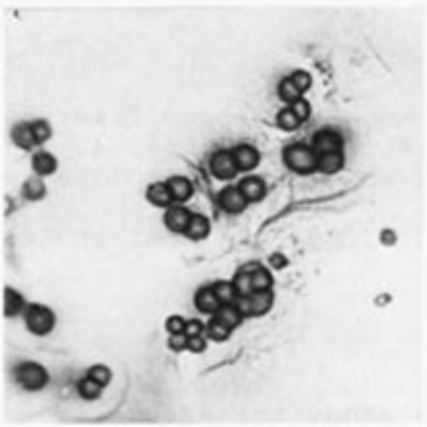


Fig. 14 Cells of *A. chroococcum* isolated from Egyptian soils (x 1 000) and E.M. (x 22 000) of the cell showing flagellation
(contributed by M.N. Hegazi)

5.3.2 Production of Azotobacterin

Since 1937, methods of azotobacterin production have undergone fundamental changes.

In one method the parent azotobacter culture was grown on an Ashby agar medium for two days. The cells were harvested and introduced into ground peat suitable for azotobacter growth; 40 mg of wet bacterial cells per kg of dry peat were used. After careful mixing, the peat was left for several days at 20-24°C. At the end of this incubation, the peat was diluted with peat powder and again incubated at the same temperature for several days, until the bacterial titre reached 10^8 cells/g peat.

With the development of growing azotobacter in submerged cultures, A. chroococcum, used in azotobacterin preparations, was also grown in fermenters and liquid azotobacterin was used. However, the shortcomings of this preparation are rapid loss of viability upon storage, and difficulties in transportation.

Accordingly, different methods and carriers were tested to obtain dry preparations of azotobacter, e.g. silica gel, lyophilization, sawdust, talcum, starch and chalk. None of these media were successful because they affected the viability of the azotobacter.

Various other methods for the production of azotobacterin were then developed in state and collective farms and research stations as well as in industrial plants in the U.S.S.R. The following is an example of a method popularized at the Microbiology Institute of the Academy of Sciences of the Ukranian S.S.R.

Well decomposed marshland peat or powdered humus with neutral or weakly alkaline pH is sieved. The growth medium comprises 1 ton of the substrate: 10 kg sugar or 20 kg molasses, 1-2 kg superphosphate, 20 kg chalk or 10 kg slaked lime, and a starter of azotobacter cells (harvested from the agar surface of 25 Roux bottles). The Azotobacterin is then produced in 2 stages. First, half the substrate is mixed with the appropriate amount of nutrients and the entire amount of starter; this is mixed well with shovels and then spread out in a 20-40 cm layer and incubated at 20-25°C. Then, after 3 days incubation, the rest of the substrate and the nutrients are added. The resultant mass is kept for a further 3-5 days, with daily shovelling. The bacterial fertilizer is then ready for use.

5.3.3 Methods of Application of Azotobacterin

The most widely used method of applying azotobacterin is by treating seed or planting material immediately (i.e. same day)prior to sowing or planting.

Azotobacter cells are grown on agar in Roux bottles and harvested with water. The bacterial suspension is further diluted with water and used to spray seeds, or in the case of potato tubers or seedlings for example, they are soaked in the suspension.

Peat and soil azotobacterin preparations are also diluted with water, and the resulting suspension is sprayed on seeds and seedlings. Peat azotobacterin is sometimes used as a top dressing on sugarbeet.

The following rates per hectare are used for different azotobacterin preparations:

i. A ar preparation: growth from 600 ml Roux bottles containing 4.6×10^{10} azotobacter cells is used to treat cereals. The growth from 350 ml Roux bottles is used for treating potato tubers and vegetable seedlings .

ii. Peat preparation: 3 kg are used to treat cereals and 6 kg to treat potato tubers and vegetable seedlings. One gramme of preparation should contain 5 x 10^7 azotobacter cells.

iii. Liquid preparations from submerged cultures: 200 ml for cereals and 500 ml for potato tubers and vegetable seedlings.

5.3.4 Effect of Azotobacterin on Plant Yield

Rubenchick (1960) reviewed 1 095 experiments in the U.S.S.R. on yield response to azotobacter. In 890 of the experiments (81%) there was an increase in yield of cereal, vegetable and industrial crops. The 1949 report of the Ministry of Agriculture of the U.S.S.R. (Table 34) shows that increases in crop yields exceeded 10% in the majority of experiments.

Table 34 EFFECT OF AZOTOBACTERIN ON CROP YIELDS IN VARIOUS REGIONS OF U.S.S.R.
(Rubenchick, 1960)

Regions	Number of experiments	Increase in yield after using azotobacter, %
	Spring wheat	
Poltava	2	5.48
Vladimir	6	12.92
	Potato	
Gorki	1	10.21
Kirov	12	23.28
Vorshilovgrad	l	23.09
Poltava	1	30.00
Vladimir	2	32.37
Leningrad	5	7.56
	Cabbage	
Vladimir	1	23.73
Kirov	1	18.48
Voroshilovgrad	3	20.76
	Barley	
Kirov	2	11.90
Leningrad	1	21.73
	Oats	
Kirov	2	24.48
Gorki	1	8.98
	Rye	
Kirov	1	14.29
Vorshilovgrad	1	16.66
Poltava	1	28.57

Another survey was made by the All Union Institute for Agricultural Microbiology of experiments carried out at a number of research institutes from 1949 to 1955 and at collective farms from 1949 to 1951 (Table 35). Again in the majority of the experiments, azotobacterin increased crop yields.

Table 35 EFFECT OF AZOTOBACTERIN ON CROP YIELDS ON COLLECTIVE FARMS IN U.S.S.R. 1949 - 1951
(After Rubenchick, 1960)

Plant	Number of experiments	Positive experiments %	Control Crop (kg/ha)	Increment (kg/ha)
Spring wheat	38	89.0	540	90
Winter wheat	5	100.0	1 040	180
Winter rye	4	100.0	660	85
Oats	26	92.0	755	110
Barley	15	86.0	905	85
Millet	3	66.6	400	70
Buck wheat	1	100.0	250	50
Corn (maize)	8	100.0	1 260	220
Foxtail millet	2	100.0	1 015	390
Seed flax	4	75.0	175	35
Sugar beet	10	100.0	11 520	1 145
Cabbage	3	100.0	10 900	3 265
Cotton	27	81.0	1 280	140
Potato	35	85.7	6 015	1 220

Original figures in centner/ha converted to kg/ha (one centner = 50 kg).

Experiments on azotobacterin in Czechoslovakia in 1954 (Czechoslovakian Academy of Agricultural Sciences) showed that yield increases for sugar beet, maize, carrot and cabbage were 39%, 15.4%, 19.2% and 2.9% respectively (Rubenchick, 1960). Studies in Romania in 1954, showed an increase of 25 to 50% in sunflower yield.

Results of studies on the response of various crops to azotobacterin outside the U.S.S.R. have been inconsistent. Increases of 11% in carrot yield, 6.25% for potatoes and 13% in the green matter of mustard have been reported in Germany. In other instances, e.g. in Switzerland, France, Denmark, Finland and the U.S.A. results have been negative.

Brown et al (1962, 1964) studied the effect of inoculation with Azotobacter chroococcum on plant growth and yield using three methods to introduce the bacterium into the soil and rhizosphere i.e. cultures applied to seeds, roots and soil. A wide range of crops was tested, e.g. spring and winter wheat, barley, lettuce, radish, cabbage, cress, carrot, spinach, sugar beet, and tomatoes. The results were variable and inconclusive.

Careful investigations using chemical and isotopic techniques have snown that heavy inoculation with azotobacter cultures fails to effect the incorporation of nitrogen into soil. The inoculated bacteria frequently die (Alexander, 1977). Nevertheless, plant responses are sometimes evident and thus explanations must be sought other than in nitrogen metabolism.

A more plausible alternative hypothesis is that species of Azotobacter produce compounds that act as plant growth regulators. Indeed azotobacters synthesize stimulatory compounds such as gibberellins, cytokinins and indole acetic acid (Vancura, 1961; Brown, Jackson and Burlingham, 1968).

Another hypothesis is based on the control of disease by azotobacter inoculation. Brown et al (1968) reviewed cases where azotobacter inoculations reduced disease, e.g. flax wilt, lodging of spring cereals, potato scab and potato blight. They indicated that it is difficult to assess the value of these reports and unlikely that all the effects are explicable by azotobacter being antagonistic towards all pathogens, although azotobacter has indeed been shown to antagonize certain bacteria and fungi, e.g. Candida albicans, Monilia and Alternaria. Indirect effects cannot be explained either, i.e. stimulation by azotobacter of some organisms antagonizing pathogens or synthesizing biologically active substances that stimulate plant development, thus enabling seedlings to grow away from the disease.

Patel (1969) investigated the effect of azotobacter inoculation on wheat and tomato rhizosphere microflora. Azotobacter became established in the rhizosphere of wheat and tomato plants and stimulated their growth. All the bacterial groups examined were more abundant in the rhizosphere than in the soil. Inoculation with azotobacter delayed the colonization of roots by bacteria, actinomycetes and fungi in the rhizosphere, but had no effect on other organisms. Inoculation did not affect the dominant root surface fungi and minor changes were not consistent.

Ridge and Rovira (1968) conducted an extensive series of glasshouse and field studies in South Australia to evaluate the inoculation of wheat with Azotobacter, Bacillus polymyxa and Clostridium pasteurianum.

The results of trials carried out in 1963 and 1964 are summarized in Table 36, giving yield relative to 100 for uninoculated plots. The Table shows that there was a much greater tendency for inoculation to increase grain yield than to decrease it. If ± 5% is accepted as a minimum meaningfull yield difference (though not necessarily statistically significant), then, of the 71 comparisons, there were 28 increases and four decreases following inoculation.

5.4 AZOTOBACTER PASPALI

This species was named by Dobereiner (1966). It associates with the grass Paspalum notatum, a subtropical grass widely distributed in South America. This organism occurred abundantly in all 75 root surface soil samples from P. notatum and in 3 out of 4 samples from the root surface of P. plicatum. Root surface soil samples from 81 other pasture plants, including more than 38 species of gramineae and 8 species of leguminosae, were all negative.

Table 36 EFFECT OF SEED INOCULATION ON GRAIN YIELDS OF WHEAT IN FIELD TRIALS
(After Ridge and Rovira, 1968)

Variety	Year	Inoculum	Site					
			A	B	C	D	E	F
Gabo	1963	Nil	100	100	100	100	100	-
		Az	138***	116***	90	107	109	-
		Bs	134***	115***	94	105	103	-
		Cl	121***	112***	92	106	102	-
		Mix	137***	121***	81	95	112*	-
	1964	Nil	100	100	100	-	100	100
		Az	105	102	100	-	100	-
		Bs	104	103	99	-	97	-
		Cl	112	101	99	-	104	-
		Mix	112	112*	101	-	105	108
Insignia	1963	Nil	100	100	100	100	100	-
		Az	98	104	104	95	111*	-
		Bs	95	102	104	104	110*	-
		Cl	99	103	106	94	109*	-
		Mix	97	96	99	104	111*	-
	1964	Nil	100	100	100	100	100	Barley 100
		Az	108	113	105	-	106	-
		Bs	112	-	102	-	106	-
		Cl	106	-	103	-	103	-
		Mix	101	104	103	-	111*	113*

Sites: A Waite Institute (red brown earth) B Parafield (red brown earth)
C Saddleworth (black soil) D Clinton (loamy terra rosa)
E Monarto (sandy terra rosa) F Meninie (deep sand)

Inoculum: Az = *Azotobacter*, Bs = *B. polymyxa*, Cl = *C. pasteurianum*

Figures represent yield relative to 100 for the uninoculated control.

Statistical significance: *, *** = significant at 5, and 0.1% respectively.

5.4.1 Potentiality of Nitrogen-Fixation

Dobereiner, Day and Dart (1972a) reviewed the characteristics of A. paspali. In the field, A.paspali becomes permanently established in the rhizosphere of four tetraploid cultivars of Paspalum notatum, but not in the rhizosphere of other, mostly diploid, cultivars, or other Paspalum species, even when artificially inoculated.

5.4.2 Location of Nitrogenase Activity

By dissecting paspalum roots into different components and measuring nitrogenase activity by acetylene reduction, Dobereiner et al (1972a) found that high activity is associated with the presence of roots. Thin sections showed that P. notatum “batatais” roots with high nitrogenase activity have a prominent mucigel layer.

Colonies of bacteria were observed on the root surface associated with mucigel which might provide the right condition for fixing nitrogen and also protect cells from being washed off the roots.

5.4.3 Amount of Nitrogen Fixed in the Field

Dobereiner et al (1972a) extrapolated that rates 15, 44 and 93 kg N/ha/year could be obtained in different locations in Brazil. This amount of nitrogen-fixation would suffice for the growth requirements of P. notatum in the field. It is also consistent with its ability to establish a good cover on poor sandy soils without any fertilizers.

5.5 BEIJERINCKIA

Azotobacter indicum was described as a new azotobacter species by Starkay and De (1939) when isolated from two Asiatic soils of low pH, e.g. 4.9 and 5.2 (Becking, 1961). Later, Derx (1950) urged that this organism be set in a new genus named Beijerinckia.

Cells are single, straight or slightly curved rods or pear-shaped, with distinctly rounded ends. Characteristically large, highly refractile lipoid (poly B-hydroxy butyrate) bodies occur at each end of the cell.

In liquid media no surface pellicle is formed, but the whole medium becomes a homogenous, highly viscous, semitransparent mass. In some species (e.g. B. fluminensis) the whole medium becomes opalescent and turbid, and adhering slime is not produced.

On agar medium, copious slime is produced and giant colonies develop with a smooth, folded or pellicated surface. The slime is extremely tough, tenacious or elastic and makes it difficult to remove part of the colony with a loop (Fig. 15).

Atmospheric nitrogen is fixed in a nitrogen-deficient medium. Molybdenum is required for nitrogen-fixation.

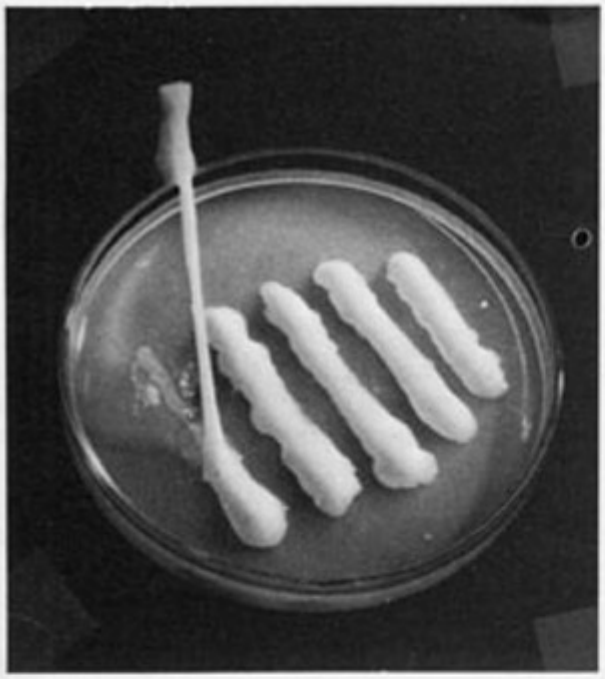
Cells are highly acid and alkaline tolerant, and will grow between pH 3.0 and 10.0.

5.5.1 Taxonomy

Four species have been recognized in the genus Beijerinckia: B. indica, B. mobilis, B. fluminensis and B. derxii (Becking, 1974a).

5.5.2 Geographic Distribution

One of the main features of this genus is its apparent restricted geographic distribution. Although the organism has been isolated from soils outside the tropics, its main distribution with respect to cell numbers per quantity of soil is in tropical soils and to a lesser degree in subtropical soils(Becking, 1977).



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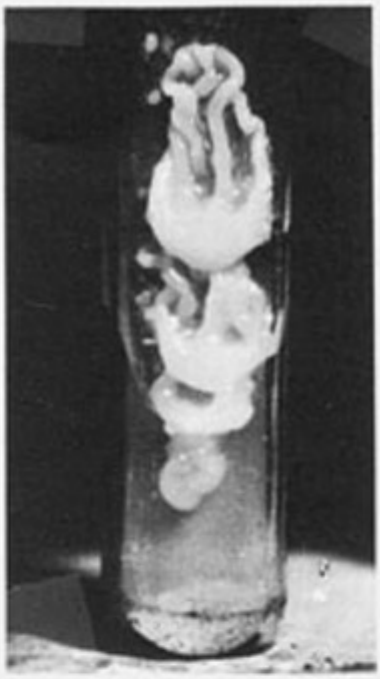

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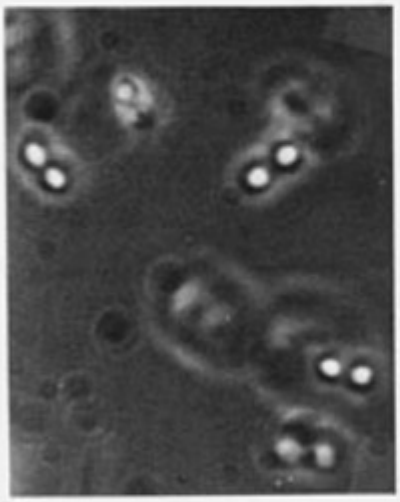


c

d

Fig. 15 *B. indica* isolated from Sudanese soils

a and b: plate and tube culture demonstrating characteristic growth of *Beijerinckia*

c and d: cells resolved by phases contrast (x 1 000); d - ultra-thin section showing 2 lipoid bodies at both poles of the cell

(contributed by M.N. Hegazi and Sonya Ayob)

5.5.3 Soil pH

Becking (1961) showed that soil samples of pH 5.0-5.4, 5.5-5.9 and 6.0-6.4 contained the greatest proportion of Beijerinckia positive samples. In growth experiments in culture medium, maximum nitrogen-fixation was obtained at pH 4.5. In some strains, the optimum pH was 4.0 and a considerable decrease in nitrogen-fixation was observed at higher and lower pH values. However, some reports indicate that Beijerinckia has been isolated from soils with pH 8.27 and 7.25.

5.5.4 Vegetation

Vegetation seems to exert an influence on the distribution and occurrence of Beijerinckia in soil. Derx (1950) noted the particular abundance of Beijerinckia in hydromorphic paddy soils. It also tends to be more abundant in the rhizosphere of the rice plant than in tree soils.

Dobereiner (1961) found that in sugarcane fields, Beijerinckia was particularly abundant along the root surface, suggesting that the root secretions in the rhizosphere promote the development of this organism in the vicinity of the roots. However, according to Hegazi et al (1979) Beijerinckia was not isolated from the rhizosphere soil or root surfaces in sugarcane fields in Egypt, where the soil pH was between 6.8-7.2.

5.5.5 Significance in Soil Improvement

Evidence has been presented by ^{15}N studies that the roots of sugarcane can fix 0.2 to 0.38 and 0.44 to 23.8 g of nitrogen/ha/day based on the values of ^{15}N uptake by non-ammended intact systems and disturbed systems, respectively (Dobereiner, 1961; Ruschel et al, 1977). A similar calculation of fixed nitrogen taken up by sugarcane leaves in intact systems gives values of 5.7 to 16.9 g of nitrogen/ha.

On the other hand, ^{15}N fixed in the soil, as tested in a disturbed system, amounted to 250 g nitrogen/ha/day, assuming a soil density of 1.3 and a 20 cm thick layer where roots are more effective, and a linear rate of growth. Assuming at least 200 days in the life of the crop, this could amount to 3.38 kg for plant fixation and 50 kg for rhizosphere nitrogen-fixation. Examination of the microbial population in this system revealed the presence of Azotobacter, Beijerinckia, Derxia, Caulobacter, Clostridium and Vibro (Ruschel and Vose, 1977).

5.5.6 Efficiency of Nitrogen-Fixation

The efficiency of Beijerinckia to fix nitrogen in pure cultures, was found to be about 16 mg N/g glucose consumed. Beijerinckia grew slowly and after 20 days only 50% of the glucose had been consumed (Becking, 1978c).

5.6 AZOSPIRILLUM

In 1922, Beijerinck reported the occurrence of nitrogen-fixing Spirillum in an enrichment culture of A. chroococcum. At first he named it Azotobacter spirillum, but afterwards, in 1925, he gave it the name Spirillum lipoferum. Becking (1963) obtained isolates able to assimilate ^{15}N which required 0.01-0.005% yeast extract to grow below the surface of semi-solid malate media. Later on, the organism was found to be common in free as well as rhizosphere soils in different climatic conditions, as indicated in Table 37. In Egypt, the occurrence of S. lipoferum was reported in association with several plants (Table 38).

Table 37 OCCURRENCE OF NITROGEN-FIXING SPIRILLUM IN ROOTS AND SOILS UNDER VARIOUS CLIMATIC CONDITIONS
(Dobereiner, 1975)

Country	Latitude	Roots[a] Number of samples examined	Roots[a] Number positive	Soils[b] Number of samples examined	Soils[b] Number positive
U.S.A.	40-48°	72	11	54	5
Brazil	28-30°	52	14	-	-
	0-23°	584	293	69	35
Senegal	15°	24	10	24	12
Gambia	14°	6	5	6	6
Liberia	6°	8	4	8	8
Nigeria	6°	20	10	18	6
Kenya (1 700 m)	2°	18	0.0	18	4

(a) Washed roots (0.5 cm) of grasses and legumes.
(b) Enrichment cultures in semisolid malate medium, which showed nitrogenase activity above 10 nano mole CH_4 ethylene/hr/culture (3ml) after 40-48 hr incubation at 33°C and contained predominantly Spirillum lipoferum populations, were considered positive.

Table 38 OCCURRENCE OF NITROGEN-FIXING SPIRILLA IN MALATE ENRICHMENTS PREPARED FROM RHIZOSPHERE SOIL AND ROOTS OF VARIOUS PLANTS
(Hegazi *et al*, 1979)

Plant		Rhizosphere soil	Ethanol treated roots	Types of spirilla prevailing
Gramineae	*Saccharum efficinarum* L.	+++	++	I,II & III
	Dendrocalamus sp	++	--	II
	Zea mays L.	+++	++	II & III
Leguminosae	*Trifolium alexandrinum* L.	++	++	I
	Glycine max Merr	+++	++	II
Malvoceae	*Gossypium barbadense* L.	+++	++	II
	Hibiscus esculentus L.	++	+++	II & III
Tiliaceae	*Chorchorus oletorius* L.	+++	--	I
Solanaceae	*Lycopersicon esculentum*	+++	++	I & II
Cruciferae	*Raphanus sativus* L.	+++	+++	I

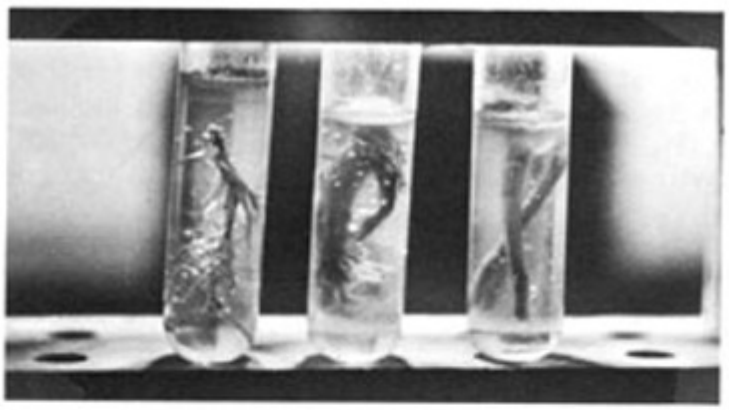
++ or +++ indicates the degree of occurrence.

5.6.1 Isolation of Spirillum lipoferum

S. lipoferum can be isolated from field grown root pieces with highly active nitrogenase activity as estimated by the acetylene reduction method (Day and Dobereiner, 1975).

These Spirillum enriched cultures form a fine white pellicle 2-4 mm below the surface of the medium (Fig. 16). The most active enrichment cultures, selected by acetylene reduction, were streaked onto plates of the same basic composition, but with calcium malate and 5 ml of 10% fresh yeast infusion per litre added. After one week, typical irregular dry white colonies, often with greenish centres, were selected, replicated into semisolid calcium malate medium with 15 ml (0.05%) yeast extract/1, and after 24 hours again checked for acetylene reduction. Purity of the most active of these isolates was checked again on agar plates before they were stored under oil in the semisolid calcium malate yeast medium.

The most characteristic forms were observed in the nitrogen-free sodium-malate medium. They are curved rods of varying sizes with a half to several spiral forms and with certain refractive lipid bodies. The cells are extremely active and the mode of motility is very characteristic (Figures 17 and 18).



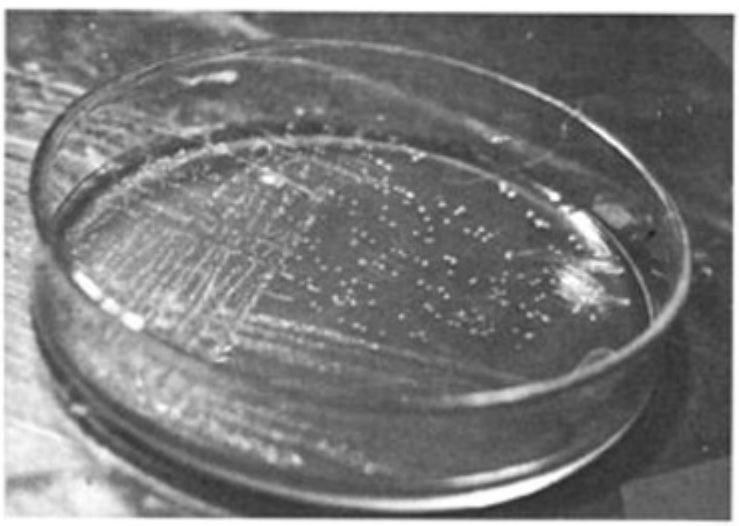


Fig. 16 Pellicles of *Azospirillum* enrichments from maize roots and colonies of *A. lipoferum* grown on malate agar (contributed by H.A. Amer and M. Eid)

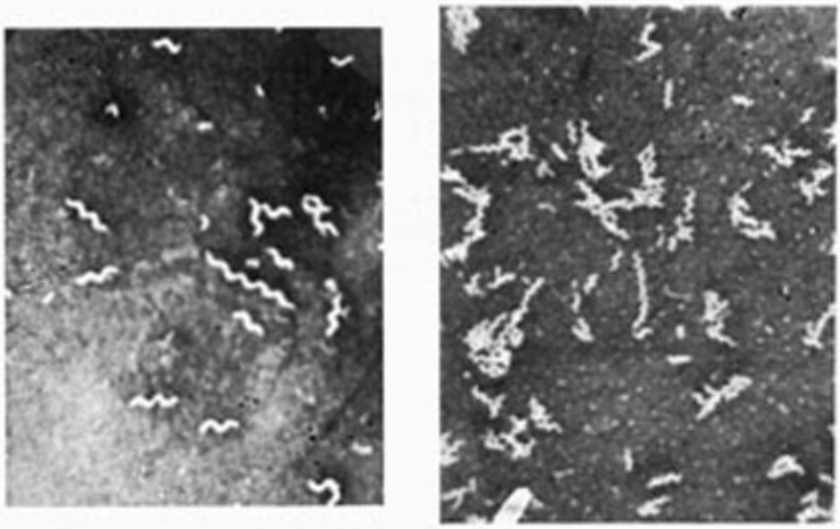


Fig. 17 Different types of *Azospirillum* sp isolated from Egyptian soils
(contributed by M.N. Hegazi, M. Monib, H. Amer, M. Eid)

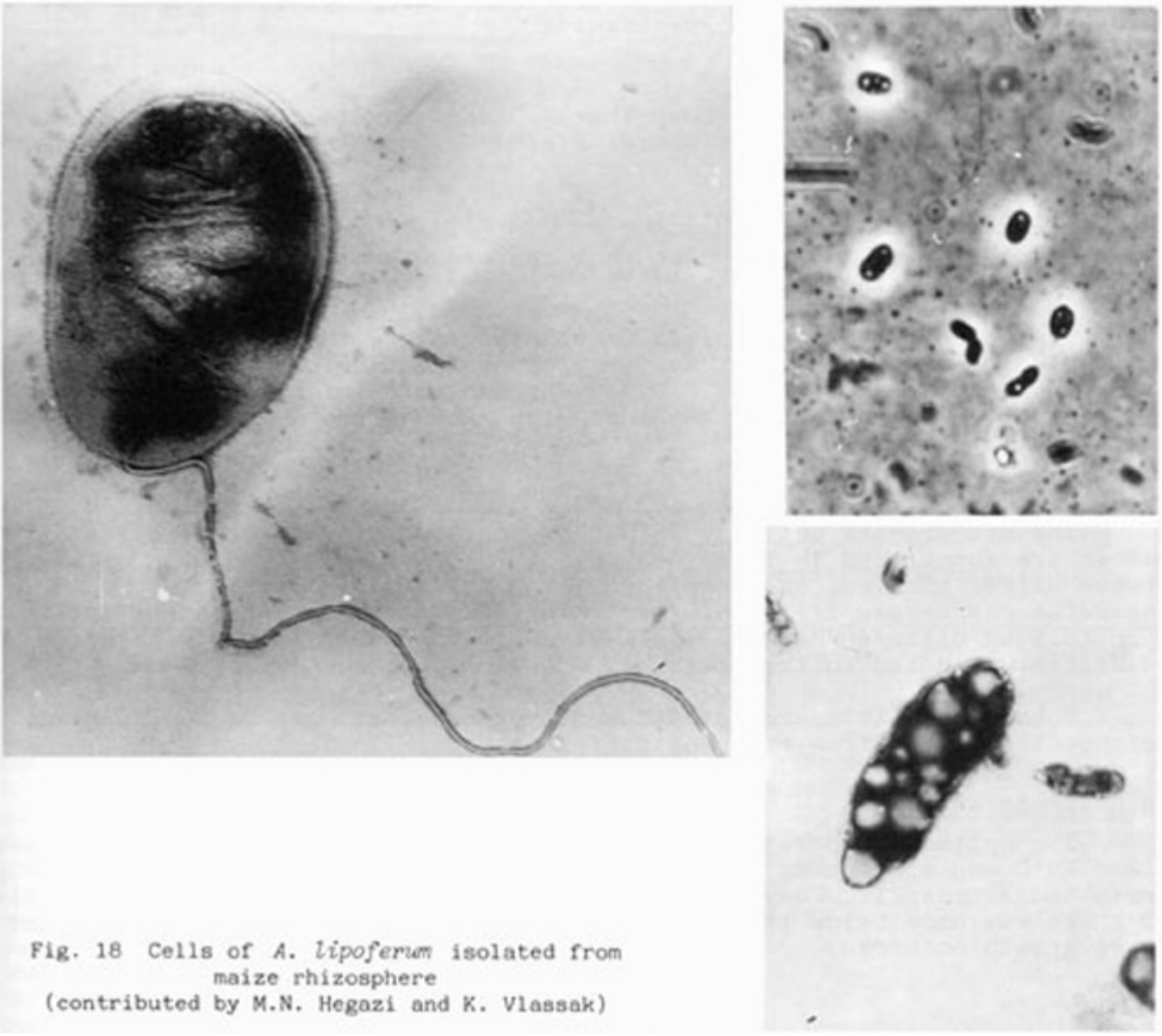


Fig. 18 Cells of *A. lipoferum* isolated from maize rhizosphere
(contributed by M.N. Hegazi and K. Vlassak)

5.6.2 Taxonomy

The taxonomic position of S. lipoferum is not yet agreed upon. Sampaio et al (1978) indicated that there are three groups of isolates of this species based on the physiological characteristics, especially nitrate reduction, as follows.

Group I (includes type strain ATCC 29145): reduces nitrate and produces gas from ammonium nitrate. Does not require biotin for nitrogen-fixation or growth; does not use glucose; catalase positive; relatively resistant to streptomycin, tetracycline, gentamycine and chloramphenicol.

Group II Does not require nitrate; requires biotin for growth on nitrogen and NH_4^+; grows on glucose; catalase negative; more sensitive to above-mentioned antibiotics.

Group III Identical to group I, except in the ability to reduce nitrate further and that it shows higher sensitivity to tetracycline.

Krieg (1977) suggested the generic name Azospirillum and named two species: A. lipoferum and A. brasiliensis.

5.6.3 Location of A. lipoferum

Accumulating evidence shows that A. lipoferum is present on the surface as well as inside the roots of maize, Digitaria and other grasses (Dobereiner, 1978; Burris, 1977).

5.6.4 Nitrogen-Fixation Potential

Studies with ^{15}N have shown that the organism is capable of fixing nitrogen by itself (Burris, 1977).

The potential of the spirillum to associate with roots to fix nitrogen was studied on an excised root system pre-incubated before the acetylene test, and on an intact plant (Burris, 1977). Higher rates of nitrogen-fixation (C_2H_2 reduction) were usually obtained with excised roots rather than from intact plants.

5.6.5 Nitrogen-Fixation Rates

The nitrogenase activity of washed roots of field grown tropical grasses are summarized in Table 39 (Day, 1975). A large variation exists between sites, species, and sampling time. The maximum values for Pennisetum purpureum, if transformed into fixed nitrogen indicate fixations of up to 1 kg nitrogen/ha/day. Activity was associated mainly with roots and little was noted in the soil.

Dobereiner (1978) reported the nitrogenase activity of S. lipoferum enrichment cultures from roots of different crops (Table 40).

5.6.6 Temperature

The optimum temperature for nitrogen-dependent growth is high, between 32 and 40°C and similar to that of organisms from tropical environments, e.g. Derxia and A. paspali. Day (1975) found that below 24°C growth was very slow and there was none below l8°C. At 42°C the nitrogenase became inactive and little growth occurred.

Table 39 NITROGENASE ACTIVITY ON ROOTS AND IN THE RHIZOSPHERE SOIL OF TROPICAL FORAGE GRASSES (Day, 1975)

		C_2H_4/g dry roots/hr nana mole	C_2H_4/g soil/hr nana mole
Brachiaria mutica	Brazil	156 - 730	0.0
B. rugulosa (Tanner grass)		5 - 148	-
Hyparrhenia rufa		17 - 29	0-0.148
Digitaria decumbens		21 - 404	0-0.349
Pennisetum purpureum		5 - 954	0-0.085
Panicum maximum		20 - 294	0-0.148
Melinis minutuflora		13 - 41	0-0.187
Cynodon dactylon		17 - 269	0-0.168
Paspalum notatum (Batatais)		2 - 283	0-0.330
Andropogon gayanus	Nigeria	15 - 270	-
Cenchrus ciliaris		16	-
Cymbopogen giganteus		60 - 85	-
Cynodon dactylon		10 - 50	-
Cyperus spp		2	-
Hyparrhenia rufa		30 - 140	-
Hypothelia dissoluta		10 - 15	-
Panicum maximum		75	-
Paspalum commersonii		25 - 30	-
P. virgatum		3	-
Pennisetum purpureum		60	-
P. coloratum		13	-
Setaria anceps		1 - 120	-
Andropogon	Ivory Coast	50 - 380	-
Brachiaria brachylopha		100 - 140	-
Bulbsylis aphyllanthoides		74	-
Cyperus obtusiflorus		30 - 620	-
C. zollingeri		50 - 160	-
Cyperus sp		1150 -1900	-
Fimbristylis sp		80 - 190	-
Hyparrhenia dissoluta		2 - 4	-
Loudetia simplex		54	-

Table 40 NITROGENASE (C_2H_2) ACTIVITY OF *S. LIPOFERUM* ENRICHMENT CULTURES OBTAINED WITH ROOTS AND RHIZOSPHERE SOIL FROM VARIOUS TROPICAL CROPS (Dobereiner, 1978)

Crop	Species or family	nana mole C_2H_4/h^{-1} culture	
		Roots	Soil
Guinea grass	*Panicum maximum* Jacq	181 sp	232 sp
Sorghum	*Sorghum vulgare* Pers.	102 sp	144 sp
Sweet potato	*Ipomoea batatas* Poir	213 sp	219 sp
Cassava	*Manihot esculenta* Crantz	74 sp	182 sp
Bracken fern	*Pteridium aquilinum* L. Kuhn	161 sp	133 sp
Goiaba (guava)	*Psidium guajava* L.	72	23
Mango	*Mangifera indica* L.	59	60
Banana	*Musa* sp	3	26 sp
Banana	*Musa* sp	13	63 sp
Coffee	*Coffea arabica* L.	2	176 sp
Coconut	*Cocos nucifera* L.	27	181 sp
Cotton	*Gossypium hirsutum* L.	15	88 sp
Sisal	*Agave sisalana* Perr.	4	119 sp
Bamboo	*Bambusa* sp	3	66 sp
Mimosa	*Mimosa pudica* L.	4	1

Mean of cultures from one sample each plant.
Numbers followed by sp are from cultures containing predominantly *S. lipoferum*.

5.6.7 pH

Nitrogenase activity is dependent upon pH, the optimum being between pH 6.8 and 7.8; there is little growth and nitrogen-fixation below pH 5.5 or above 9.0.

5.6.8 Oxygen

Control of the partial pressure of oxygen is critical in growing S. lipoferum under conditions requiring nitrogen-fixation. If ammonia is supplied as a source of nitrogen, the organism grows as a typical aerobic one. When the organism is fixing nitrogen, it operates as a micro-aerophilic organism (Burris, 1977).

5.6.9 Response to Inoculation

Smith et al (1975) used a liquid inoculant of S. lipoferum for several crops. The cultures were grown in 10 litre fermenter bottles using nitrogen-free medium with 0.5% malate as the energy and carbon source. The mixture was aerated with a mixture of 5% oxygen and 95% nitrogen. A temperature of 40°C gave faster growth than 30°C and was used when rapid growth was desired. For inoculation, an aqueous solution of the culture was added to the rows to give about 8×10^6 cells per metre of row and watered into the soil by sprinkler irrigation immediately after application. Table 41 shows the rates of nitrogen-fixation in the roots of various crops.

Table 41 FIELD WORK ON THE INOCULATION OF VARIOUS CROPS WITH S. LIPOFERUM
(Smith et al, 1975)
Acetylene reduction (n mole g^{-1} dry root h^{-1})

	No. of lines	Range	Lines above 50 nana mole %	Lines above 100 nana mole %
Zea mays	63	0- 377	28	21
Sorghum sp	51	0-1974	88	69
Pennisetum americanum	5	0- 522	60	40
Chloris gayana	36	0- 180	17	5
Cenchrus ciliaris	3	0- 163	33	33
Panicum maximum	7	0- 115	29	14
Digitaria sp	21	0- 32	0	0
Paspalum notatum	3	0- 16	0	0
Hemarthria altissima	15	0- 16	0	0

Smith et al (1978) also reviewed several years work on the response of various crops to inoculation with S. lipoferum. During the first year (1974) inoculated Panicum maximum and Digitaria decumbens produced 80 and 61% more protein respectively than uninoculated controls. In 1975, inoculation produced significantly higher protein and dry matter yields in Pennisetum americanum and P. maximum (Table 42). Projected yields using regression analysis, of both crops indicated that about 40 kg N/ha/year were replaced by inoculation. Although overall production was lower during 1976, yield responses due to inoculation were similar to those of 1975 in Panicum maximum. Response to inoculation was enhanced at higher rates of nitrogen fertilizer application e.g. 80 and 120 kg N. Changes in population of S. lipoferum were monitored using fluorescent immunological analysis 10 months after inoculation. Inoculated plots gave significantly higher counts than the control plots but the latter had a considerable number of bacteria. The bacterial populations in uninoculated plots were relatively constant over the fertilizer treatments, whereas in inoculated plots higher counts were obtained as more nitrogen was applied.

Table 42 DRY MATTER FORAGE YIELDS INOCULATED WITH S. LIPOFERUM COMPARED TO UNINOCULATED
(Smith et al, 1975)

Fertilizer N rate kg/ha	Dry matter yield inoculated	Dry matter yield uninoculated	Increase from inoculation
	kg/ha	kg/ha	
Pennisetum americanum (pearl millet)			
0	4 750	5 300	-550
20	5 820	6 070	-250
40	7 350	6 040	1 310*
80	9 140	7 880	1 260*
Panicum maximum (guinea grass)			
0	9 960	10 750	-790
30	15 550	13 460	2 090*
60	17 610	15 560	2 050*
120	20 020	19 470	550
Cenchrus ciliaris (buffle grass)			
0	1 640	2 030	-390
20	2 938	2 642	292
40	3 097	2 544	548
80	3 625	2 974	651

* Significant at 5% level

Rinaudo et al (1975) reported that inoculating variety IR 8 rice grown in a mixture of sand and alluvial soil (9 parts of sand : 1 part of soil) with Azotobacter chroococcum and Beijerinckia camargensis did not alter significantly the nitrogenase activity, but inoculation with Spirillum lipoferum increased the activity significantly (Fig. 19).

In greenhouse experiments, Burris (1977) reported very little response of maize inoculated with Spirillum lipoferum in terms of yield or total nitrogen in the plants. Furthermore,in field trials in sandy soil in the Wisconsin River valley, a slightly higher percentage of the uninoculated plants gave a better total yield than the inoculated plants, but the differences were well within the statistical variations of the experiment.

In a different heavier soil near Madison, Wisconsin, Burris (1977) ran a field trial using 19 different varieties or species of plants (Table 43); 14 of them showed a positive response and the other 5 a negative response to inoculation with S. lipoferum.

Again the results are not statistically significant, but they were definately in a positive direction toward benefit from S. lipoferum inoculation. Use of these data to calculate yields and increased nitrogen on a per hectare basis indicates a possible increase in total nitrogen of 2 to 5 kg/ha during a growing season.

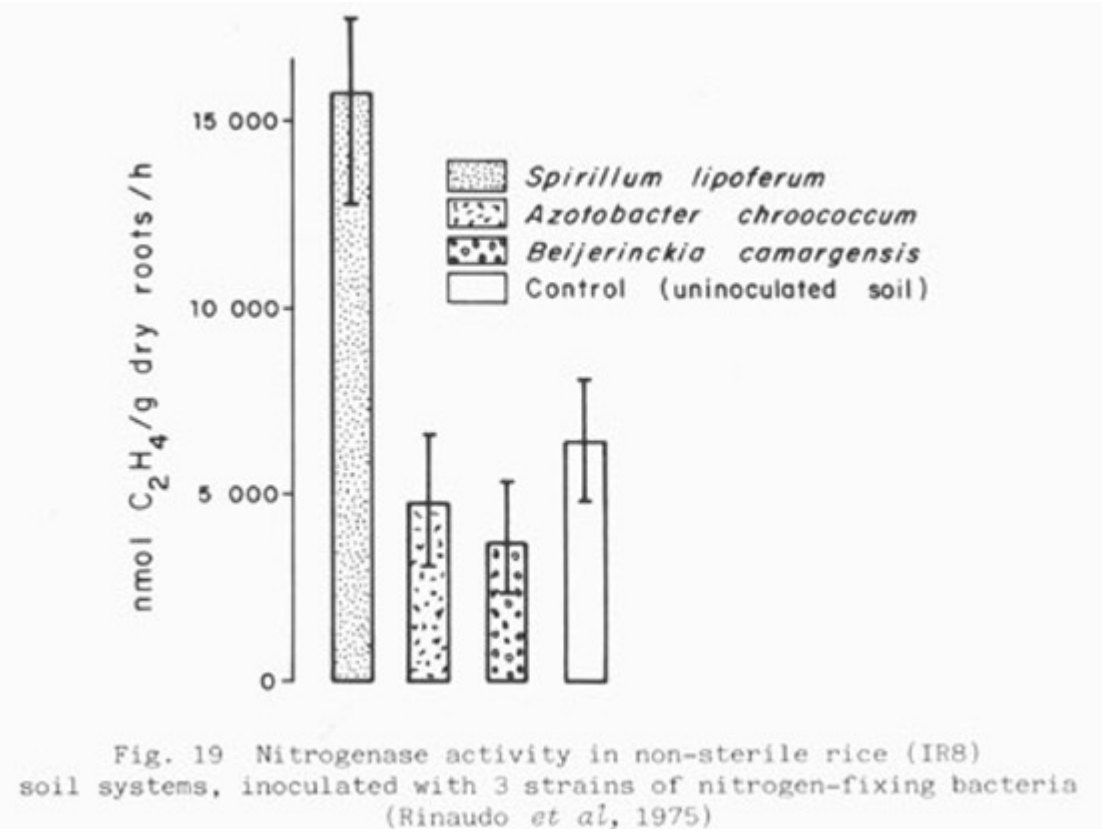


Fig. 19 Nitrogenase activity in non-sterile rice (IR8) soil systems, inoculated with 3 strains of nitrogen-fixing bacteria (Rinaudo *et al*, 1975)

Table 43 FIELD TRIALS ON 19 VARIETIES OR SPECIES OF MAIZE AND OTHER PLANTS WITH AND WITHOUT INOCULATION WITH S. LIPOFERUM (After Burris, 1977)

Cultivar	Inoculated		Uninoculated	
	g	% N	g	% N
W 59 E	163	0.99	169	1.02
W 153 R	225	1.25	246	1.16
W 64 A	240	1.19	243	1.23
Wis 900	376	1.12	422	1.07
Field corn	519	1.09	318	1.07
Sorghum	385	0.37	478	0.40
Sorghum (Sudan)	592	0.56	356	0.45
W 729 D	223	1.23	223	1.10
W 182 E	205	1.13	178	1.08
W 629 A	113	1.39	103	1.45
W 513	205	1.28	145	1.25
W 61	235	1.19	218	1.13
W 629	237	1.15	223	1.10
W 438	289	1.11	266	1.12
A 654	211	1.26	223	1.19
Gold Rush	372	1.18	302	1.12
Golden Cross	439	1.15	390	0.96
Golden Beauty	329	1.10	286	1.12
White Cloud	320	1.06	262	1.06

6. AZOLLA

6.1 INTRODUCTION

Azolla is a symbiotic algal association that grows on the surface of water (Fig. 20). It has long been grown in lowland rice fields, both as green manure to enrich the field and as a feed for domestic animals.

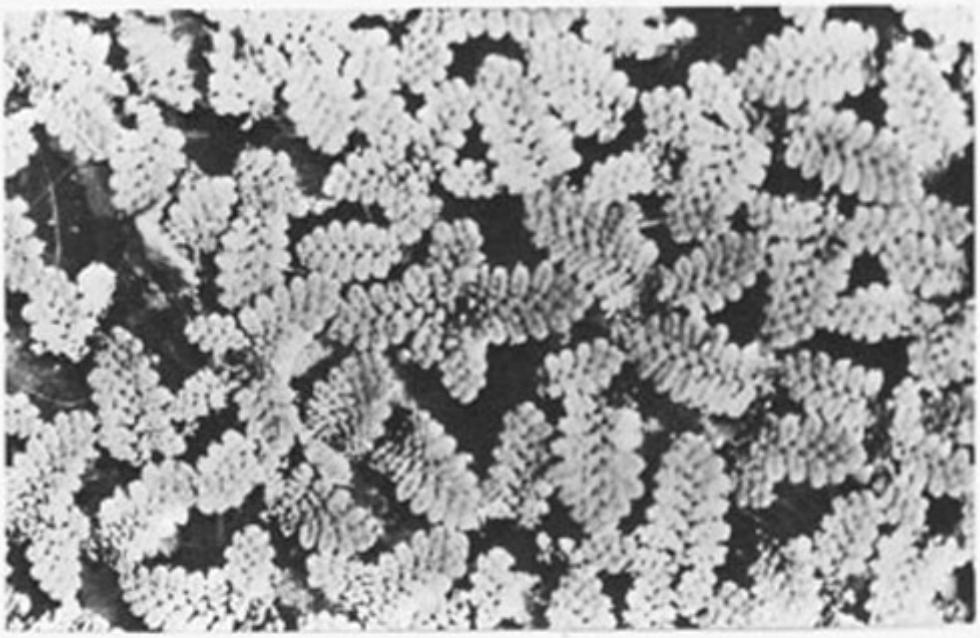


Fig. 20 *Azolla* fern floating on water (contributed by I. Watanabe)

Unlike chemical fertilizers and other nitrogen-fixing plants, azolla does not contaminate the environment nor does it consume the photosynthates of the rice plants. In China acclimatization has made it possible for it to live through the high temperatures of summer and the cold temperatures of winter and now a total of more than 1.3 million ha of rice are cultivated there with azolla.

6.2 TAXONOMY

The genus Azolla belongs to the family Azollaceae (Salvinaceae) of the order Salvinales of the Division Pterodophyta (Lumpkin and Plucknett, 1978). The six existing forms (Becking, 1979) are usually divided into two sections:

Section I Azolla (or Euzolla). This section contains four species:

- A. filiculoides Lam., found throughout the Americas and widely distributed elsewhere,
- A. caroliniana Willd., found in eastern U.S.A., Mexico and the West Indies,
- A. mexicana Presl., northern South America to British Columbia and eastward to Illinois,
- A. microphylla, western and northern South America to southern North America and the West Indies.

Section II Rhizosperma. This section covers only two species:
- A. pinnata R. Br., widespread in the eastern hemisphere, tropical and southern Africa, southeast Asia, Japan and Australia,
- A. nilotica Decaisne. A large species occurring on the Nile in Africa.

Tuan and Thuyet (1978) reported that Van Liem (in 1962) described three varieties of azolla: green, purple and red (Table 44). It has been generally accepted by farmers and research workers that green azolla is the most resistant to high temperature, the red to low temperature, the purple one to acidity and the red is also resistant to salinity.

Table 44 CHARACTERISTICS OF AZOLLA VARIETIES COMMON IN VIETNAM (Tuan and Thuyet, 1979)

Characteristics	Azolla varieties		
	Green	Purple	Red
Size of leaf clusters	medium	great	very great
Leaf arrangement	dense	dense	sparse
Colour of ventral lobes	white	pinkish white	pinkish
Colour of dorsal lobes			
- favourable conditions	light green	dark green	dark green
- unfavourable conditions	orange	purple	dark red

6.3 AZOLLA-AZOLLA ANABAENA ASSOCIATION

In 1873 Strasburger published a monograph on Azolla in which he indicated that there was almost always a blue-green alga belonging to the Nostocaceae present in cavities of the leaves (Moore, 1969).

i. The fern

Azolla pinnata consists of a floating, branching rhizome, with small, alternate overlapping leaves and simple roots which hang down into the water (Fig. 21). Each leaf is bilobed. The upper thick green lobe contains the green pigment chlorophyll and the lower thin lobe is pink, without chlorophyll. The anthocyanin pigment is also found in the upper lobe under certain conditions which gives it a reddish brown colour. The lower surface of the lower lobe remains in contact with the water.

Roots occur at branch nodes on the ventral surface of the stem. They have root hairs and a sheathed root cap that falls off with age. The roots contribute more to total fresh weight in plants lacking the symbiont than to those containing it (Peters, 1976).

In nature (but also in the greenhouse), and under a combination of appreciable light intensity and low temperature, azolla populations may develop a brick-red colour. The nature of the anthocyanin compound formed has recently been identified, by chromatographic analyses, to be two pigments. The main pigment is a luteolinidin glycoside and there is pigeninidin glycoside in smaller quantities (Peters et al, 1977).

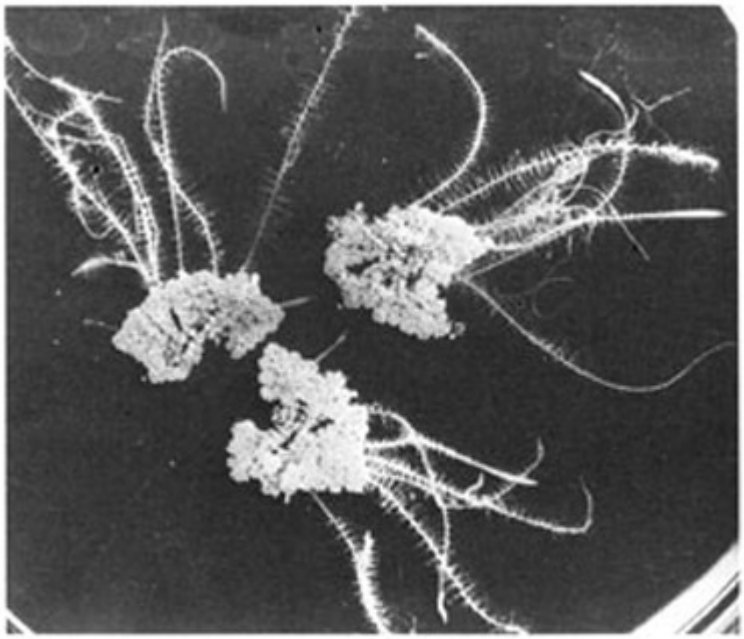


Fig. 21 *Azolla pinnata* fern
(contributed by P.K. Singh)

ii. The symbiont

Examination of the Azolla-Anabaena association shows that the algal symbiont grows in association with the fern at two different topographic sites, i.e. in leaf cavities of the aerial upper lobe of the bilobed azolla leaves and in close connection with the apical meristem of the plants (Becking, 1978 a).

Anabaena azollae Strasburger is the only species mentioned in symbiotic association with Azolla (Fig. 22)

Pockets of the symbiont, with approximately the same size and shape as found in the individual leaf chambers, have been isolated by digestion of the host tissues with cellulytic enzymes. These pockets contain the symbiont and associated hair cells within the envelope. When the pockets are ruptured to release the symbiont, the hair cells remain associated with the envelope material (Peters et al, 1979). These authors postulated that the hair cells and envelope may be associated with the exchange of metabolites between the host tissues and symbiont present in mature cavities.

Many workers have isolated the algal symbiont from A. pinnata (Venkataraman, 1962), A. mexicana (Holst, 1976), A. filiculoides (Ashton and Walmsley, 1976) and A. caroliniana (Wieringa, 1968; Newton and Herman, 1977) . However, as no successful re-establishment of the symbiotic state between any of these isolates and the algal-free fern plants has been reported (Peters et al, 1979) , caution must be maintained when referring to any of the isolates as the algal symbiont until this association can be reproduced.

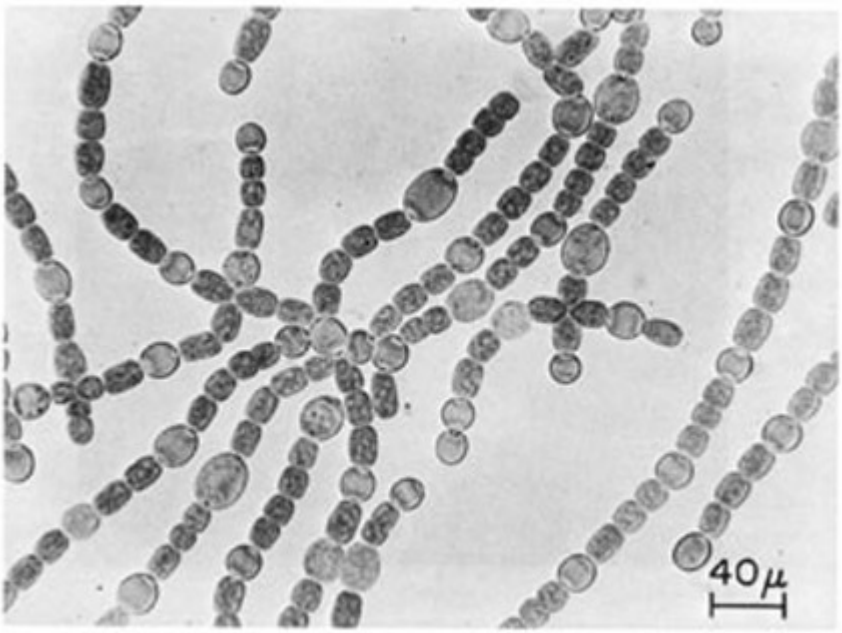


Fig. 22 Blue-green algae *Anabaena azollae* (contributed by G.A. Peters)

6.4 REPRODUCTION

Propagation of azolla is always through vegetative reproduction. However, azolla is heterosporous and produces both mega- and micro- sporangia within the sporocarps which are formed by the lower lobe initial of the first leaf of a branch. Details of this sexual cycle have been reported by several workers (Becking, 1978 a; Peters, 1978). Significantly, the symbiont maintains its association with the host plant during the sexual cycle, an outline of which is given in Fig. 23.

6.5 NITROGEN-FIXATION

Although azolla can utilize nitrogen present in its aquatic environment, the algal symbiont is capable of meeting the entire nitrogen requirement of the association. Nitrogen-fixation by Azolla-Anabaena symbiosis has been demonstrated indirectly by the use of nitrogen-free nutrient solution, acetylene reduction-gas chromatography and assay of H_2 production, and directly by the use of ^{15}N tracer method (Lumpkin and Plucknett, 1978).

Nitrogen can be supplied to the association by nitrogen-fixation or by absorption from an aqueous medium, or by any combination of the two without the loss of nitrogenase activity (Peters et al, 1976). Nitrogenase activity by A. azollae is protected by the fern from combined nitrogen in the medium. Peters and Wayne (1974) found that even after six to seven months of growth in a medium containing nitrogen, there was still appreciable nitrogenase activity in the azolla fronds.

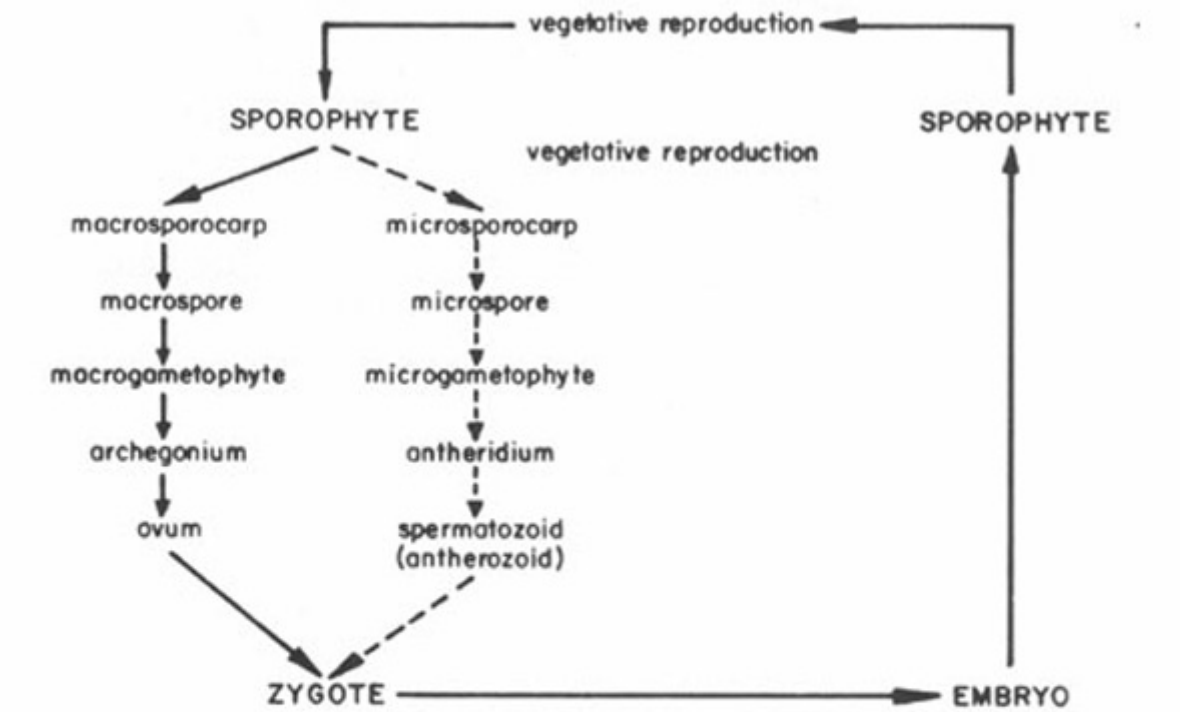


Fig. 23 Life cycle of *azolla*: solid arrows represent the continuation of the association with *anabaena azollae*

Talley et al (1977) measured the nitrogenase activity of A. mexicana and A. filiculoides in a greenhouse and under controlled environment conditions (26°C and 300 microeinsteins/m^2/sec). The results were respectively 800 + 25 and 773 + 38 n mole acetylene reduced per g dry wt/min. Midday acetylene reduction rates obtained in the field for fully developed covers of these two ferns have peak values of about half those found in the laboratory.

Ashton (1974) found that acetylene reduction for A. filiculoides was 960 n mole/g/dry wt/min at about 50% full sunlight and at 25°C. Rates at full sunlight were only 60% of the optimum, implying an upper limit for acetylene reduction in full sunlight of about 575 n mole/g dry wt/min.

Becking (1978a) showed that A. pinnata had a doubling time of 2.6 days, a growth rate of 0.308 g/g per day and nitrogen-fixation of 9.86 mg N/g dry weight per day.

Watanabe et al (1977) has reported that under the most favourable conditions the doubling time of A. pinnata is 3 days, and it may fix 7.8 mg N/g dry weight per day.

6.5.1 Effect of Combined Nitrogen

The symbiont retains significant nitrogenase activity when azolla is grown with combined nitrogen. When azolla was grown for 85 days with nitrate or urea as a nitrogen source the rate of acetylene reduction was decreased by approximately 30% compared to the control grown on nitrogen (Table 45). Prolonged growth on nitrate or urea (6-7 months) resulted in a 90% decrease in the rate of acetylene reduction (Peters and Mayne, 1974). Ammonium chloride was inhibitory to acetylene reduction and it did not support the growth of the fronds as much as other sources.

Table 45 EFFECT OF COMBINED NITROGEN SOURCES ON ACETYLENE REDUCTION (Peters and Mayne, 1974)

Nitrogen source	Rate of C_2H_4 produced nana mole/mg total chlorophyl/min	S D	Number
N_2	27.1	5.3	7
Nitrate	18.5	3.8	6
Urea	18.4	2.4	7
Ammonium	10.3	0.2	10
N_2 dark control	0.09	0.08	4

Chu (1978)[1] indicated that when the nitrogen concentration (as ammonium nitrate) is increased nitrogen is reduced. Under a weak light of 1500 lux and with 1000 ppm ammonium nitrate, activity of nitrogenase is completely checked, but it retains some activity with 10 000 lux of light (Table 46). With a weak light of 1500 lux and 100 ppm ammonium nitrate azolla grows better. When the concentration is increased, the rate of growth decreases. However, under strong light (10 000 lux) and 10 ppm ammonium nitrate, the yield increases slightly. If the concentration is increased, the yield decreases.

Table 46 EFFECT OF VARIOUS CONCENTRATIONS OF NITROGEN ON THE YIELD AND NITROGEN FIXATION CAPACITY OF AZOLLA (Chu, 1978)2

	Nitrogen conc. ppm NH_4NO_3	Yield ratio of azolla (after 5 days) %	Capacity of acetylene reduction C_2H_4 nana moles/mg fresh wt/day
Weak light	0	100.0	0.8710
1 500 lux	10	100.0	0.6105
	100	116.7	0.3333
	500	90.0	0.165
	1000	90.0	0
Strong light	0	100.0	4.534
10 000 lux	10	109.8	3.432
	100	74.1	2.228
	500	68.5	1.452
	1000	62.9	0.858

[1]Personal communication

[2]This Table is extracted from the working paper by Dr Chu for the IRRI Symposium on Nitrogen and Rice.

Note. The base culture solution was Checkiang Agr. 6302 free from N; light was from tungsten iodic lamp, 28°C.

6.5.2 Nitrogen-Fixation Efficiency

The azolla association is capable of significant light-dependent nitrogenase H_2 evolution (Newton, 1976; Peters, 1978). In an argon atmosphere, hydrogen production by the association and by the isolated alga was measured to be 21.7 nana mole H_2/mg/chlorophyll (a)/min, and 88.6 nana mole/H_2/chlorophyll (a)/g. (Peters et al, 1976). Peters (1978) indicated that when azolla was grown on N_2, efficiency values were 0.94-0.99 while those obtained when the frond was grown on nitrate, were 0.60-0.84. Newton (1976) reported efficiency values of 1 which indicates no H_2 production in air took place and 0.89 when azolla was grown with nitrate. These results suggest that azolla is a candidate for biosolar H_2 production when grown on nitrate (Peters, 1978).

6.5.3 Nitrogen-Fixation and Conversion Factors

The ratio of C_2H_2 reduced to that of nitogen fixed was found to decrease as N_2 pressure increased to 0.8 atm. Ratios of C_2H_2 fixed at partial pressure of N_2 of 0.3, 0.6 and 0.8 atm were 3.2, 2.0 and 1.7 for the frond, but they were 4.4, 3.0 and 2.5 for the blue-green algae, respectively (Peters, 1978).

6.5.4 Release of Fixed Nitrogen by the Algae

Anabaena azolla, when isolated from the azolla's cavities and incubated under N_2, releases ammonia into the incubation medium (Peters, 1976). No ammonia is released during incubation under Ar or N + 2% Co. ^{15}N experiments showed that, in general, 50% or less of the nitrogen fixed is incorporated into the symbiont. Newton (1976) indicated that fixed nitrogen constituted up to 50% of the total nitrogen in the free nitrogen pools of the azolla association when it was grown in the absence of combined nitrogen, and that there were substantial levels of glutamine, glutamate and cystathionine.

Some azolla species secrete nitrogenous compounds into their aquatic environment. Shen et al (1963) reported that a Chinese variety of azolla (Whole River Bed) released 12-14% of its fixed nitrogen into the water. Brill (Peters, 1977) indicated that a specimen of A. mexicana excreted about 20% of its fixed nitrogen ammonia.

Because of the ability of azolla to fix nitrogen and to pump out ammonia, it is hoped that this system can be employed as a living biosolar-driven system for ammonia production in the future.

6.5.5 Photosynthesis of Azolla

Both the azolla plant and the algal symbiont removed from the plant are photosynthetically competent. The azolla contains chlorophyll(a) and (b) while the alga contains chlorophyll(a) and phycobilins. Both organisms contain carotenoids. On the basis of three separate methods of determination, the algal partner has been estimated to contribute 15 to 20% of the association's total chlorophyll (Peters et al, 1979).

6.5.6 Growth of Azolla

Talley et al (1977) studied the growth rates of A. filiculoides and A. mexicana that are indigenous species in the Sacramento Valley of Central California. A. filiculoides is ubiquitous in agricultural drainage channels and other calm water surfaces. Healthy ferns growing in open water have a biomass doubling time of less than 7 days during the summer. When fully developed A. filiculoides may possess a biomass of over 180 g/dry weight/m^2 and contain 58 kg N/ha. A. filiculoides is frost hardy and can withstand temperatures as low as 5°C without suffering apparent harm.

A. mexicana grows rapidly during the summer (with a doubling time of 2-8 days) but the fern is not resistant to frost. Maximum biomass development which has been observed in the field is 98 g dry weight/m^2, representing a nitrogen cover of 29 kg/ha.

Azolla populations grown with rice during the summer have rapid initial growth and reach peak biomass 28 days after inoculation. The subsequent decline in biomass is rapid, especially in A. mexicana, implying a significant nitrogen loss from the biomass to the aquatic environment.

Espinas and Watanabe (1976) determined the growth rate of Azolla pinnata. The results (Table 47) showed that azolla doubled its weight in 3-5 days during the first week. In two weeks, approximately 30-40 kg N/ha had accumulated in the azolla tissue.

Table 47 NITROGEN INCREASE AND GROWTH RATE OF AZOLLA
(Espinas and Watanabe, 1976)

Date inoculated	Nitrogen increase, weeks kg/ha			Growth rate, weeks, kg/ha	
	0-1	1-2	0-2	0-1	1-2
1 200 cm^2 tray					
11/7	12.9	23.1	36.0	3.7	5.6
12/2	9.8	17.8	27.7	4.6	5.9
1 884 cm^2 tray					
11/7	14.5	16.3	30.3	2.9	7.4
12/2	10.4	12.2	22.4	3.6	8.3

6.6 TOTAL NITROGEN INPUT ESTIMATES OF AZOLLA

Estimates of the total nitrogen input of azoZZa in paddy soils are rather variable. Some examples are given below.

Becking (1972) calculated, upon C_2H_2 reduction assays of A. pinnata, a maximal input of 335-670 kg N/ha/year. Saubert (1949) reported a biomass of 310 kg N/ha/year. Moore (1969) estimated a potential fixation value of 100-160 kg N/ha in three to four months. Olsen (1972) found an input of 95 kg N/ha with A. filiculoides over 3 months (June-August) which would give 380 kg N/ha/year if growth and nitrogen fixation should proceed throughout the year. Watanabe (1977) measured an accumulation of 330 kg N/ha in 220 days. Talley et al (1977) measured (in the Sacramento Valley of Central California), nitrogen accumulation of over 50 kg N/ha within 35 days with A. filiculoides, which means an annual increase of 500 kg N/ha. High figures are cited from Vietnam, e.g. 1 000 kg N/ha, and China, e.g. 92.7-151.8 kg N/ha in 1.5 months (Shen et al, 1963).

These estimates are based in most cases on unlimited continuous growth of azolla in areas of open water with optimal conditions, whereas, in agricultural practice, the growth of azolla is discontinuous. Correcting the values by taking into account the cover and reduced nitrogen fixation at night, a figure of 103-162 kg N/ha would be acceptable (Becking, 1976).

6.7 ENVIRONMENTAL FACTORS AFFECTING AZOLLA

6.7.1 Water

Water is a fundamental requirement for the existence of azolla. At a relative humidity of less than 60% azolla becomes dry and fragile; and when it is completely dry it dies. Although azolla can grow on a wet mud surface or wetted peat litter, it prefers to grow in a free floating state on a calm water surface, and may be found on ponds, canals, lakes and also on some slow-moving rivers. It survives only in the presence of sporocarps (Becking, 1979).

6.7.2 Light

While some reports indicate that high intensity of light enhances the growth of azolla, others show that the optimal light intensity for growth is 15-18 kilo lux and that at higher intensities growth and photosynthesis are inhibited. At pH 5, high intensity of light enhances growth but at pH 6 and 7, it inhibits growth (Tuan and Thuyet, 1979).

Ashton (1974) observed that both the growth rate and nitrogenase activity of A. filiculoides change with increasing light. In South Africa, it was found that growth increased with increasing light intensity, up to a maximum of 50% sunlight (i.e., about 49 000 lux). Further increases in light intensity decreased the growth rate. In 25% sunlight the growth rate was higher than in full sunlight. The nitrogenase activity followed the same pattern i.e. highest at 50% sunlight and lower at both 25 and 75% sunlight.

Peters (1976) showed that nitrogen (C_2H_2) fixation was already saturated at the low light intensity of 4 840 lux in the intact association of azolla,but only at 2 156 lux in the isolated alga. The low light requirement of the symbiont can be explained as an ecological adaptation because of the lower light intensities that reach the symbiont as a result of the screening effect of host pigments in the intact association.

Brotonegoro and Abdulkadir (1976) studied the effect of prolonged shading on the activity of A. pinnata. The exposure of plants shaded for 5 hours to light intensities of 16-57% of full sunlight did not affect the nitrogenase activity of the association, but a shading of 16% of full sunlight for 10 hours reduced the nitrogenase activity of the intact plants to 30% of that of the control plants.

A 12 hour dark period caused the nitrogen (C_2H_2) reduction rate to remain constant at 25-50% of that in light. This demonstrated that, in the dark, the intact association can supply the algal symbiont with endogenous reductant accumulated during the previous light period (Becking, 1978a). Talley et al (1977) showed that A. mexicana has a greater tolerance to high light intensity than A. filiculoides.

6.7.3 Temperature

Several workers have reported various ranges in temperatures, but the optimum temperatures for azolla spp seem to be between 18-28°C (Tuan and Thuyet, 1979).

The optimum ranges of temperature for A. caroliniana, A. filiculoides (Ashton and Walmsely, 1976) and A. pinnata (Watanabe et al, 1977) appear to be between 25 and 30°C. Although A. mexicana will not tolerate frost, A. filiculoides will survive it (Talley et al, 1977).

6.7.4 pH

Several workers have indicated that the optimal pH for azolla growth varies from 4.5 to 7.5. They have shown that the response of azolla to pH depends on many factors such as temperature, light intensity and the presence of soil and soluble iron (Tuan and Thuyet, 1979) (Table 48).

Table 48 INFLUENCE OF pH ON AZOLLA GROWTH IN A RICE FIELD WATER SOLUTION UNDER DIFFERENT CONDITIONS (RELATIVE DRY WEIGHT) (Tuan and Thuyet, 1979)

	Relative dry weight %					
pH	Normal condition	High temperature	Low light intensity	With iron	High temperature	With soil
5	100	100	100	100	100	100
6	87	61	93	110	92	121
7	82	54	86	108	88	119

Singh (1977) indicated that using aoils of different pH revealed that the fern grew better in the slightly acid soils of Cuttack (ph 5.9) but grew badly in Hyderabad soil of pH 8.1 (Table 49). The very acid soils of Pokkali (pH 2.9) and Kattampalli (pH 3.6) did not support growth and the plants died within 2 and 10 days in the two soils, respectively. The pH of standing water on Cuttack, Hyderabad, Kattampalli, and Pokkali soils, was 6.6, 8.1, 3.4 and 3.2 respectively, after 15 days.

Table 49 GROWTH OF AZOLLA IN SOILS OF VARIED pH (Singh, 1977)

Soil collections	Initial pH	pH after 15 days of floodin		Inoculum fresh wt, g	Growth after 15 day fresh wt.g
		Soil	Standing water		
Cuttack (CRRI)	5.9	5.9	6.6	2	15.1
Hyderabad	8.1	8.0	8.1	2	6.5
Kattampalli	3.6	5.6	3.4	2	0
Pokkali	2.9	3.0	3.2	2	0

6.7.5 Season and Soil Conditions

The growth of azolla on alluvial neutral soil, rich in phosphorus, is better than on acid soils poor in phosphorus. But on acid soils, the yield in summer is higher than that in other seasons (Table 50) (Tuan and Thuyet, 1979).

Table 50 AZOLLA GROWTH IN DIFFERENT SEASONS AND TYPES OF SOILS
(Tuan and Thuyet, 1979)

Seasons and Temperature range	Alluvial pH 7.0 P_2O_5 0.09%		Alluvial pH 5.5 P_2O_5 0.57%		Degraded light pH 6.1 P_2O_5 0.045%	
	Fresh wt t/ha/5 days	Growth rate	Fresh wt t/ha/5 days	Growth rate	Fresh wt t/ha/5 days	Growth rate
Spring						
20-25°C	11.19	0.245	8.53	0.141	9.13	0.165
Summer						
27-29°C	10.63	0.225	9.70	0.190	9.66	0.186
Autumn						
26-29°C	9.36	0.170	9.22	0.168	7.78	0.111
Winter						
17-22°C	7.19	0.090	9.34	0.173	9.51	0.180

6.7.6 Inoculum Density

Tuan and Thuyet (1979) indicated that as a general rule the optimum inoculum density is 500 g/m^2 For specific cases the following ranges of densities were regarded as appropriate.

i. in paddy fields for the production of green manure: 250-500 g/m^2
ii. in the inoculum production plots or for the production of compost: 500-750 g/m^2
iii. in the production of animal feed, or where land is limited: 750-1 000 g/m^2

However, it appears that densities can vary considerably in different countries. In Vietnam, Thuyet and Tuan (1973) recommended a planting rate of 500 g/m^2 for A. pinnata which can increase to a density of 1 000-1 600 g/m^2 in winter and 1 000-1 400 g/m^2 in summer. In India Singh (1977) recommended rates ranging from 400-1 000 g/m^2. In the U.S.A. an inoculum of 500 g/m^2 of A. mexicana and A. filiculoides is recommended (Talley et al, 1977).

6.7.7 Nutrition

i. Phosphorus

Rains and Talley (1978) indicated results of experiments with A. filiculoides and A. mexicana to ascertain their requirement of phosphorus. Azolla grown in a phosphorus-deficient solution decreased or ceased growth, became red in colour and developed curled roots (Watanabe and Espinas, 1976). A. filiculoides grown as a fallow crop in the spring might require as much as 60 kg P_2O_5/ha, either as a single application or as a split treatment of 30 kg P_2O_5/ha each time. Maximal production of A. mexicana was obtained with a split application of 15 kg P_2O_5/ha in equal doses at 1, 10 and 21 days after inoculation. The higher yield thus obtained seemed to result from less stimulation of competing green or blue-green algae. At higher levels of phosphorus fertilization, competing organisms proliferated and may have inhibited azolla development (Rains and Talley, 1978).

ii. Iron

The addition of 1.5 kg Fe/ha was sufficient to eliminate the deficiency in paddy water (Rains and Talley, 1978). The critical concentration of iron for azolla growth was found to be 20µg Fe/l. The availability of iron as a nutrient to plants is affected by pH. When the pH is high (alkaline) iron is precipitated as hydroxide and is therefore less available to plants. Unavailability of iron causes yellowing of the ferns (Espinas and Watanabe, 1976).

Iron is usually present in culture medium at levels of 0.01 to 3.0 mg/l. It can be added in the form of concentrated iron citrate or EDTA (or versenate) to give a final concentration of 2-5 ppm iron, (Becking, 1979). In the field, 9.1 kg Fe/ha are enough for a 5 month irrigation period for rice with a water flow rate of 1.4 1/sec (Becking, 1972)

iii. Other mineral nutrients

Azolla plants require all the macro-nutrients (phosphorus, potassium, calcium, magnesium, iron) and micro-nutrients (molybdenum, cobalt) . Vanadium and tungsten are reported to be a suitable substitute for molybdenum (Moore , 1969) .

Espinas and Watanabe (1976) compared the growth of azolla in phosphorus, potassium, calcium and magnesium deficient media, a nitrogen ammended medium and in a complete nitrogen-free medium as control (Table 51) . Fresh weight, nitrogen content and acetylene reduction activity measured when harvesting the azolla in the P and Ca deficient media gave far lower values than those in the control, K and Mg deficient media and the N-ammended medium. Fresh weight and nitrogen content data for azolla in a potassium deficient medium are still lower than those in the control medium but acetylene reduction activity is twice as much or more than that in the control medium. This means that the nitrogen-fixing ability of potassium deficient azolla is kept active.

Table 51 FRESH WEIGHT, TOTAL NITROGEN AND ACETYLENE REDUCTION ACTIVITY OF AZOLLA IN P, K, C AND Mg DEFICIENT MEDIA AND N-AMMENDED MEDIUM
(Espinas and Watanabe, 1976)

Treatment	Fresh weight g/plate	Total N kg/ha [1]	C_2H_2 : C_2H_4 assay nana mole/g/ha
-N	35.40 ± 2.35	44.58 ± 0.21	340 ± 200
-N -P	8.15 ± 0.65	7.36 ± 0.22	12 ± 0
-N -K	11.60 ± 2.00	10.95 ± 1.95	830 ± 230
-N -Ca	3.41 ± 0.34	2.20 ± 0.11	0
-N -Mg	30.30 ± 1.70	34.28 ± 0.73	441 ± 539
+N	12.50 ± 0.90	19.31 ± 1.86	259 ± 119

[1] Plate size was 314 cm^2

[2] The contaminated green algae were not completely removed

[3] Average ± standard deviation

6.7.9 Pests of Azolla

Azolla is frequently attacked by lepidopterous and dipterous insects, which are common in the summer and in rainy seasons (Singh, 1977; 1978), but insect occurence is low when the morning water temperature remains below 20°C. The larvae of insects, mostly Chironomus, Pyralis and Nymphulus (Thuyet and Tuan, 1973) roll the leaves of plants together and feed on them, causing brown patches in the azolla cover (Singh, 1979). Other pests reported from different countries are reviewed by Lumpkin and Plucknett (1978) and among them are mites, weevils, aphides and moulds.

Controlling pests is most important, otherwise the entire crop of azolla can be damaged in a few days. These insects can easily be controlled by carbofuran at the rate of 2-5 kg/ha. A smaller quantity (100-500 g) of carbofuran mixed with the inoculum for one hectare can protect the crop and encourage fern growth. Singh (1979) found that application of the insecticides BHC, sevidol and carbofuran did not adversely affect azolla or its symbiont. Chinese literature recommends the use of BHC and DDT (Lumpkin and Plucknett, 1978).

6.7.10 Pesticides

Becking (1979) tested 8 compounds in concentrations usually applied in agricultural practice: carbofuran (100 mg/1), dimecron (80 mg/1), diazinon (40 mg/1), thiodin (40 mg/1), 2,4-D (amine) (67 mg/1), DDT (50 mg/1), dithiocarbamate (33 mg/1) and MCPA (67 mg/1). Nitrogenase activity was assayed with the C_2H_2 reduction test in 70 ml serum bottles, and incubated at 28°C at a light intensity of 12 000 lux. In a 48 hour incubation period, no significant decrease of the C_2H_2 reduction rate was observed, possibly because the algal symbiont living in the leaf cavity of the aerial leaf was not in direct contact with either the nutrient solution containing chemical, nor the vascular system of the plant, so that the substance could not reach the symbiont suring short-term exposure.

Cohn and Renlund (1953) showed that A. caroliniana present in a canal could be controlled and eliminated by spraying with 1 000 to 10 000 ppm of 2,4-D. Diquat and paraquat was effective at l.0 ppm (Blackburn and Welden 1965). Paraquat was also effective at a concentration of 0.56-2.24 kg/ha (Matthews, 1963). Diesel oil was also toxic to azolla when applied as a foliar 1:1 spray with water (Oosthuizen and Walters, 1961).

6.8 CONTROL OF AZOLLA PESTS

Chu (1978) summarized methods recommended in China to control azolla pests as follows:

i. intermittent drainage of rice fields is good for azolla and rice, and favours the natural enemies of azoZZa pests, such as ants and frogs;

ii. soil and azolla can be disinfected by using 4.8 kg/ha (8 catties) of carbofuran at the last harrowing; 50% fenthion or 1 500 dilutions of 50% fenthion may be used to soak mother azolla for 5 min;

iii. insecticides: it is important to know the characteristics of pests before deciding on the kind and quantity of insecticide to use. It is better to use different insecticides in rotation or mixtures of them, or to use them in combination with fertilizers. A list of suggested insecticides is given in Table 52.

Table 52 SOME PRINCIPAL INSECTICIDES FOR CONTROL OF AZOLLA PESTS (Chu, 1979)

Name of insecticide	Concentration ppm	Method of application	Pests	Effect
90% trichlorfon	1 000	Spraying	Pyralis sp	Quite good
			Bagous sp	" "
80% dichlorvos	3 000	"	Pyralis sp	" "
			Bagous sp	" "
			Nymphula[1]	No effect
50% cartap	1 000-l 500	"	Pyralis sp	Quite good
			Nymphula[1]	" "
50% xenitrothion	800-2 000	"	Nymphula[1]	" "
			Polypedilum[2]	
50% malathion	2 000	"	Nymphula[2]	" "
			Pyralis sp	" "
25% phosmet	2 000	"	Pyralis sp	" "
50% fenthion	800	"	Polypedilum[2]	" "
carbofuran	30-l00	Spraying or 4-5 kg/ha mixed with soil	Pyralis sp	Fairly good
			Nymphula sp	" "
			Polypedilum sp	" "
Tea seed cake	30	Splashing	Radix swinhoer[3]	

[1] *Nymphula* tarbata Butler
[2] *Polypedilum* juinoense Hauber
[3] *Radix swinhoer* H. Adams

6.9 FUNGAL DISEASES

Rymnaea causes grey spots on the surface of the leaves. The disease is widespread at temperatures higher than the optimum. To control the disease spraying with diluted defusit (1 part defusit to 250 parts of water) is recommended after 7-10 days, using 1 125 l/ha of diluted mixture. A mixture of equal proportions of trichlorfon (diptrex) and malathion (both at 0.1 to 0.2%) is also used to control the disease. The disease known as "damping off" occurs at high temperatures and high plant densities and is controllable by thinning and ploughing-under the crop (FAO, 1977).

6.10 ROLE OF AZOLLA IN AGRICULTURE

Azolla plays a significant role in agriculture in certain parts of the world, where it is used as green manure and as animal feed, or grown with rice. Another potential use of azolla is to improve quality of water. However, in other parts of the world, it is still regarded as a nuisance and a weed.

6.10.1 Azolla as Green Manure

Singh (1979) has outlined the method used by farmers in India to grow azolla as a green manure. Fresh azolla should be spread on the surface of standing water (5-10 cm deep) at a rate of 1 000 kg fresh weight/ha and 8-20 kg P_2O_5/ha should be applied. Mixing 100-500 gm/ha of carbofuran with the inoculum protects the azolla.

Between 10-20 days, when azolla covers the whole field, the plant should be incorporated into the soil with a rotary hoe or other implement, and the rice seedlings should then be transplanted into it. Azolla dies gradually if left unincorporated on the soil surface.

If there is not sufficient water to irrigate a large area, small plots of fallow land can be used for azolla cultivation and the plants can then be gathered weekly, leaving some inoculum for further growth. About 5-10% of the land will produce enough organic matter to fertilize the entire area in 2-3 months.

Fresh or decomposed azolla may be used as a top dressing at a later stage of plant growth.

In China, azolla is grown on harvested rice fields or fallow land between the first and second crop. When it is fully grown on the surface water, it is turned into the soil with a small tractor with rotavator. The remnants are left to propagate further for use as manure. The process is repeated several times.

Soil mixed with azolla can be used directly in the fields where it grows or can be dug out to use as fertilizer elsewhere. It is estimated that in this way 66m^3 of compost material per mou (1 000 m^3/ha) can be obtained. The results of experiments have shown that 10 tonnes of comnpost mazerial generally increase rice yield by 470 kg/ha.

In the United States, Rains and Talley (1978) cultivated Azolla filiculoides in 22 m^2 plots at a rate of 50 gm/m^2. The azolla inoculated covered less than 10% of the plot area and contained 1.5 kg/ha. Within 18 days, it covered the entire plot and the nitrogen had increased. After 35 days in inoculated paddies, the azolla cover attained a biomass of 1 700 kg (dry weight) /ha containing 52 kg N/ha. The azolla was mature at 35 days and further experiments revealed a steady decline in nitrogen content and a steep decline in acetylene reduction capacity. It was also shown that 40 kg N/ha added as azolla produced almost the same yield response from rice as 50 kg N/ha added as ammonium sulphate.

6.10.2 Azolla Grown in the Field

Subudhi and Singh (1978b) reported a nitrogen-fixing rate of 7.5 mg N/day/g dry weight of azolla in 10 days incubation.

When 0.1-0.4 kg inoculum per m^2 was sown in the field (Singh, 1978) 8-15 t green matter per ha was obtained in 7-20 days and 333 t/ha, which contained 840 kg N (Table 53) and 20 t dried compost, was produced annually in breeding plots. A thick layer of azolla ensured about 30-40 kg/N/ha because it contained 4-5% N on a dry weight basis (0.2 to 0.3% on a fresh weight basis).

Shen et al (1963) compared the nitrogen-fixation ability of “red azolla” with alfalfa (Medicago sativa) and soybean (Glycine max). They reported that 1.5 months of azolla cultivation increased the nitrogen content of the soil to a level equal to that produced by a crop of soybeans, but to only 40% of the level produced by alfalfa. Their calculations were based on alfalfa, soybean and “red azolla” nitrogen contents of 2.87, 2.90 and 3.50%, respectively (dry weight basis).

The yield of azolla varies according to the locality and species of azolla. Table 54 summarizes data obtained by several workers.

Table 53 YIELD OF *AZOLLA* AND NITROGEN IN THE FIELD AND CONCRETE TANKS IN TERMS OF FRESH WEIGHT (Singh, 1979)

	Mean water temperature °C		AZOLLA (t/ha)					
			FIELDS			TANKS		
	day	night	amount inoculated	amount harvested	increase	amount inoculated	amount harvested	increase
Monthly average yield	31.6	24.6	13.6	41.4	27.7	11.8	35.5	26.8
Annual average yield			164.1	497.5	333.4	141.0	461.7	321.1
Annual nitrogen yield			0.41	1.25	0.84	0.35	1.16	0.8

The data were collected from 32 plots with a total of 256 m^2 in the field and from 12 tanks with a total of 82.8 m^2. The azolla crop was collected four times a month over 20 months (June to January, 1978).

Table 54 YIELD OF AZOLLA SPECIES IN DIFFERENT LOCALITIES

Species	Yield/t/ha Fresh	Dry weight	N/kg/ha	Reference
A. pinnata (India)	37.8	2.78		Gopal, 1967
A. mexicana (U.S.A)		0.98	24-45	Talley et al, 1977
A. filiculoides (U.S.A)		1.8-2.57	58-105	" " " "
A. filiculoides (Denmark)		3	90	Olsen, 1972
A. pinnata (Asia)	8-10		25-50	Thuyet and Tuan, 1973

6.10.3 Crop Response to Azolla

Singh (1979) showed that a layer of azolla covering a one hectare rice field contains about 10 t of green matter and provides about 25-35 Kg N/ha. This amount can be doubled by growing a second azolla layer after the first one has been incorporated or gathered. Pot (Fig. 24) and. field experiments conducted in both seasons (Rhabi and Kharif) over 3 years with high yielding rice varieties (IR 8, Vani, Supriya, Ratna, Jaya, Kalinga-2, Pusa 2-21) and cultivars (CR 1005, CR 181-5, CR 188-10, SG-1) revealed that the use of azolla increased plant growth, number of tillers, grain and straw significantly (Table 55).

Fig. 24 Crop response to *Azolla* inoculation (from left to right): *Azolla* incorporated, *Azolla* left floating and Control (no *Azolla*, no manure) (contributed by P.K. Singh)

Although green manuring with 10 t of azolla per ha is as efficient as a basal application of 30 kg/N, a split application of N fertilizer was found to be slightly superior. Grain yield increased by as much as 54% when 10-12 tonnes of azolla were incorporated in the soil or allowed to decompose without incorporation.

Table 55 EFFECT OF AZOLLA ON YIELD OF IR 8 AND SUPRIYA RICE VARIETIES DURING RABI 1977 (Singh, 1978)

	No. of panicles per m^2		Wt. of panicles g/m^2		Grain yield kg/ha		Straw yield kg/ha	
	IR 8	Supriya	IR 8	Supriya	IR 8	Supriya	IR 8	Supriya
Control	339	434	745	548	4 722	3 489	3 607	2 571
Azolla, t/ha	430	545	880	825	5 918	5 125	4 643	3 786
Increase %	27	26	18	51	25	47	29	47

6.10.4 Availability of Azolla Nitrogen

The mineralization of azolla nitrogen and its incorporation with flooded soil was faster at room temperature when incubated at 24°C (± 2°C). About 56% of N as ammonia was released in 3 weeks of incubation at 24°C, whereas 80% was released at room temperature (Singh, 1979). Watanabe et al, (1979) reported that 62-75% of the azolla N available to the rice crop was slightly less than the N from ammoniacal fertilizer.

6.10.5 Azolla Cultivated under Rice Plants

The beneficial effects of azolla grown under rice plants depend on several factors as follows (Chu, 1979).

i. The variety of the fern: well.-grown vigorous plants usually contain 3.4% dry matter and 25-32% organic carbon and have a C : N ratio of 10. Such green manure decomposes quickly in the rice field to supply available nitrogen to the growing rice plants.

ii. The variety of the rice: rice varieties with a long growing season generally give better yields than those with short ones because the longer growth period permits greater nutrient absorption.

iii. The type of soil: the addition of green manure to soils of high fertility usually increases crop yield by 9.9-27% but in soils of low fertility a 13.3-42.7% increase in rice yield can be expected.

iv. Cultural practice: turning azolla under by hand or machine gives better results than natural decompostion.

In the U.S.A. it appears feasible to grow azolla with rice provided proper management is applied (Talley et al, 1977). The density of the rice crop and the timing of the cultivation of azolla with rice are important factors. In experiments rice yields from dual cultivation of A. filiculoides and rice were 25% higher than those from unfertilized controls. The yield response was equivalent to the addition of 10 kg/ha as ammonium sulphate. With A. mexicana, rice yields were about 3 times those of controls without azolla when 40 and 80 kg N/ha were applied as ammonium sulphate.

6.10.6 Place of Azolla in Rice Rotation in Vietnam

In Vietnam, azolla is considered as a winter crop like potatoes, vegetables, maize and soybean. The place of azolla in the rotation is summarized by Tuan and Thuyet (1979) as follows:

i. spring rice (February-June) - early summer rice (July-late October) - maize, soybean, or multiplication of azolla.
ii. spring rice (February-June) - medium summer rice (July-November) - potatoes or vegetables.
iii spring rice (February-June) - late summer rice (July-November) - azolla as winter crop.

6.10.7 Double Narrow Row Method

Chu (1979) has described a double (narrow) row method of rice cultivation with azolla between the double rows of rice plants. This enables the azolla to grow for most of the year and hence increases the rice yield.

The spacing between the double rows of rice plants is 53-66 cm, and between the narrow rows of rice plants the spacing is 13 cm. The distance between the clusters (hills) of rice in the rows is 6.5 cm. The azolla is grown in the wider space between the double rows of rice plants (Fig. 25).

Table 56 shows that with this system an area of 2 ha produced an average rice yield (i.e. with two crops) equivalent to 15.65 tonnes/ha. The average yield of azolla was 133 tonnes/ha. The total nitrogen fixed (estimated by the Kjeldahl method) was 602-618 kg/ha and 733-829 kg/ha within 10-12 months for A. pocheng and A. filiculoides respectively.

Note that the above weights and measures expressed in metric units are approximate and have been converted from the original Chinese units using the following relationships: 1 Chinese catty = 0.6 kg
1 " inch = 3.3 cm
1 " mou = 0.066 ha

Fig. 25 Double narrow row method of rice cultivation (contributed by L.C. Chu)

Table 56 YIELDS OF RICE AND *AZOLLA* IN PADDY FIELDS WITH *AZOLLA* CULTIVATED MOST OF THE YEAR, POCHENG, FUKIEN, 1977
(After Chu, 1978)

Experimental units	Experimental period for cultivating *Azolla*		Yield tonnes/ha (two crops)		Highest Yield tonnes/ha		
	Area ha	Dates	*Azolla*	Rice tonnes/ha	*Azolla*	Early Rice	Late Rice
Wukeng Production Brigade	0.40	March 6 - Dec. 22	217	19.6	226	9.7	13.9
"Red flag" Production Brigade	0.38	April 7 - Dec. 23	202	18.3	231	8.0	12.8
Shi-peh 4th Production Brigade	0.28	March 1 - Sept. 18	117	13.5	163	8.0	8.7
Shin-Shi 2nd Production Brigade	0.13	April 19 - Sept. 19	113	15.9	113	6.5	10.1
Ling Fuaug 2nd Production Brigade	0.16	April 3 - Sept 9	85	15.4	85	8.2	9.0
Shui Nam Production Brigade	0.63	April 26 - Sept 2	64	14.0	111	7.1	9.1
Average Total	1.99	184 days	133	16.1			

Note The metric units have been converted from the original Chinese units as follows: 1 Chinese mou = 0.066 ha
1 Chinese catty = 0.6 kg
1 tonne = 1 000 kg

6.10.8 Recommendations for Azolla Cultivation

Chu (1979) recommends the double row method of azolla cultivation with rice outlined above (section 6.10.7).

The rice plants should be of the compact type, with straight narrow leaves, hard stems, big ears, plenty of grains and medium tillering capacity. (The same spacings as indicated in section 6.10.7 should be used).

The parent azolla should be grown in shallow water first until nearly harvest time for the rice, when the water depth should be increased. When the rice plants are fully grown use additional irrigation water, or drainage, as necessary to ensure optimum growth of the azolla.

Pests and diseases must also be controlled as necessary.

6.10.9 Nutrients for Azolla During Summer

Chu (1979) has indicated that during the summer nitrogen-fixation by azolla decreases. It appears therefore that fertilizers and micro-elements should be applied. Table 57 gives the results of applying fertilizers and micro-elements, which shows that all nutrients stimulated (fresh weight) production of azolla, but nitrogen fertilizers reduced nitrogen-fixation.

Table 57 EFFECT OF VARIOUS FERTILIZERS DURING THE SUMMER PERIOD UPON THE PROPAGATION AND NITROGEN-FIXING CAPACITY OF AZOLLA (Chu, 1979)

Fertilizer	Increase after 10 days cultivation g fresh wt	Increase %	Acetylene reduction n moles/ C_2H_4 /mg/fresh wt/day	Increase %
Macro-elements				
Ammonium sulphate 1%	29.85	162.7	3.58	34.1
Superphosphate 1%	37.45	204.1	10.96	104.3
K SO l%	26.75	134.5	11.87	112.9
Control	18.35	100	10.5	100
Micro-elements				
Sodium molybdate 1 ppm	2.96	197.3	11.13	114.4
Ferrous sulphate 500 ppm	3.01	200.7	10.73	110.3
Control	1.5	100.0	9.73	100

6.10.10 Azolla and Rice Yields

Yields from rice grown with A. mexicana were 550/kg/ha greater than for A. filiculoides and rice, and 850 kg/ha more than the controls. The amounts of A. filiculoides and A. mexicana used in the experiments were respectively 30 and 38 kg/ha. Incorporation of 60 kg N/ha into the soil. as decomposing A. filiculoides increased rice yields by 1 470 kg/ha over yields of unfertilized controls (i.e. 112%). Combining A. filiculoides as a green manure in the plots produced an increase of 2 700 kg/ha over controls (i.e. 216/), compared with increases of 292 and 1 470 kg/ha respectively for separate treatments with A. filiculoides as a dual crop and green manure (Talley et al, 1977).

Moore (1969) cites yield increases of 14, 17, 22 and 40% in four separate experiments where A. pinnata was grown together with rice.

Espinas and Watanabe (1976) evaluated the results of added phosphorus midseason,puddling to incorporate the azolla, and azolla inoculation on straw and on grain yields (Table 58). In plots with azolla inoculation, its incorporation and phosphorus, the grain and straw yield was far higher than in plots without these treatments. The effect on straw yield of midseason puddling was significant, and over-shadowed the effect of azolla inoculation.

Table 58 STRAW AND GRAIN YIELD OF RICE VARIETY IR 30 TREATED WITH PHOSPHORUS, PUDDLED AND INOCULATED WITH AZOLLA
(Espinas and Watanabe, 1976)

Treatments P U A	Straw Yield kg/ha	Grain Yield kg/ha
- - -	1 018 d	1 478 d
- + -	1 363 bc	2 358 ab
- - +	1 542 ab	1 832 c
- + +	1 316 bc	2 155 abc
+ - -	1 233 cd	1 860 c
+ + -	1 305 bc	2 245 bc
+ - +	1 378 bc	2 020 bc
+ + +	1 644 a	2 534 a

P U A: P = phosphorus, U = puddled, A = azolla
In each column, the means followed by a common letter are not significantly different at 5% level.

Scientists in the Peoples Republic of China (Department of Agriculture, 1975) reported rice yield increases of 0.4 to 158% with an average of 18.6% from the results of 422 field experiments. Experiments in Sri Lanka (Kulasooriya and de Silva, 1977), indicated that 32% more grains per panicle were produced from azolla and rice than in the control.

6.10.11 Azolla as Feed

Azolla can be used as animal feed. E.g. it can be fed to pigs fresh or fermented. The fodder contains about 1.3% crude protein and is a good green roughage similar to sweet potato vines. It is estimated that 660 m^2 (1 mou) of good green azolla provides sufficient roughage for 10 to 20 pigs (1 ha is required for 150 to 300 pigs).

Azolla harvested from the field should also provide good quality forage. Moore (1969) estimated that it contains 23% protein, 4.4% crude fats, 9.5% crude fibre and 6.4% starch (dry weight).

The chemical composition of azolla (Singh and Subudhi, 1978) is presented in Table 59. Protein content is high but ash and calcium contents are similar to commercial feed.

Based on feeding experiments to leghorn chicks, Singh and Subudhi (1978) reported that fresh azolla can substitute for 20-25% of commercial feed (Table 60). The increase in weight of birds was noticed when they were fed with azolla along with normal diet. There was no mortality of chicks when these were fed with fresh azolla.

Table 59 CHEMICAL COMPOSITION OF AZOLLA
(Singh and Subudhi, 1978)

Constituents	% (dry wt)
Ash	10.5
Crude fat	3.0-3.36
Crude protein	24 - 30
Nitrogen	4 - 5
Phosphorus	0.5-0.9
Calcium	0.4-1.0
Potassium	2.0-4.5
Magnesium	0.5-0.65
Manganese	0.11-0.16
Iron	0.06-0.26
Soluble sugars	3.5
Crude fibres	9.1
Starch	6.54
Chlorophyll	0.34-0.55

Table 60 EFFECT OF VARIOUS FROPORTIONS OF AZOLLA ALONG WITH COMMERCIAL FEED ON THE BODY WEIGHT OF CHICKS (FOUR CHICKS FOR EACH FEEDING GROUP)
(Singh and Subudhi, 1978)

Age of birds in weeks	Mean weight / bird/week, g.			
	100% feed	50% feed+ 16% azolla	75% feed+ 12.5% azolla	100% feed+ 5% azolla
7	155	120	120	149
8	193	151	149	188
9	234	179	181	245
10	278	201	252	335
11	320	231	312	398
12	373	264	416	480
13	433	309	454	544
14	494	344	518	614

6.10.12 Azolla as Weed

Because of its rapid vegetative reproduction azolla will cover a water surface very quickly. Cohn and Renlund (1953) reported that A. caroliniana covered a canal in New Jersey from bank to bank for five miles and the blanket was so dense that it was impossible to row a boat through it. Moore (1969) reviewed cases where azolla is considered a weed e.g. in paddies of the Philippines, in Hawaii, in Japan and in South Africa.

Control of azolla as a weed is acheived by using diesoline, either undiluted or mixed with water 1:1 (Oosthuizen and Walters, 1961).

6.10.13 Azolla as a Means of Improving Water Quality

High concentrations of nitrates and phosphates are the major contributors to the deterioration of water quality. Rains and Talley (1978) suggested that azolla could be a good candidate for the biological treatment of waters. It grows rapidly, and can fix molecular nitrogen, even in the presence of nitrate, which can be easily removed.

6.10.14 Other Potential Uses of Azolla

In additon to its potential for rice production, fresh azolla is a good food and when cooked can be consumed as a leafy vegetable. Fresh azolla or its compost can also be used for the benefit of field crops and vegetables. Decomposing azolla has also been used successfully in gobar gas plants. (Singh, 1978).

7. NODULATED NON-LEGUMINOUS PLANTS

The nodulated non-leguminous plants are perennial angiosperms which bear nitrogen-fixing root nodules. The first evidence that such plants fix nitrogen was obtained by Hiltner in 1896 who studied Alnus glutinosa (Stewart, 1976). Detailed knowledge of the morphology, physiology and bio-chemistry of these nitrogen-fixing plants has become available through the work of several investigators (Becking, 1977; Bond, 1974; Quispel, 1974; Akkermans, 1978).

7.1 THE MACRO-SYMBIONT. THE PLANTS

The orders, families and genera known to be nodulated are presented in Table 61 (Becking, 1977). The distribution of these nodulated non-legume angiosperms is given in Table 62 (Stewart, 1976). The species known to bear nodules are compiled by Bond (1976).

Table 61 CLASSIFICATION OF NON-LEGUMINOUS NITROGEN-FIXING ANGIOSPERMS
(Becking, 1977)

Order	Family	Genus	Number of symbiotic species. In parenthesis number of species
Casurinales	Casurinaceae	Casuarina	18 (45)
Myricales	Myricaceae	Myrica	20 (35)
		Comptonia	1 (1)
Fagales	Betulaceae	Alnus	33 (35)
Rhamnales	Elaeagnaseae	Elaeagnus	14 (45)
		Hippophae	1 (3)
		Shepherdia	2 (3)
	Rhamnaseae	Ceanothus	31 (55)
		Discaria	2 (10)
		Colletia	2 (12)
Coriariales	Coriariaceae	Coriaria	13 (15)
Rosales	Rosaceae	Dryas	3 (4)
		Purshia	2 (2)
		Cerecarpus	3 (20)

Table 62 NUMBER OF SPECIES AND DISTRIBUTION OF NODULATED NON-L LEGUMINOUS ANGIOSPERMS
(Stewart, 1976)

Genus	No. of species	Distribution
Casuarina	45	Australia, tropical Asia, pacific islands.
Myrica	35	Tropical, sub-tropical, temperate regions.
Alnus	35	Europe, Siberia, North America, Japan, Andes.
Dryas	4	Arctic, mountains of north temperate zone.
Cercocarpus	20	North America.
Purshia	2	North America.
Coriaria	15	Mediterranean, Japan, New Zealand, Mexico, Chile.
Discaria	10	South America, New Zealand, Australia.
Ceanothus	55	North America
Elaeagnus	45	Asia, Europe, North America.
Hippophae	3	Asia, Europe.
Sheperdia	3	North America.
Aktostaphyles	70	North-West and Central America, Asia.

7.2 THE ENDOPHYTE. THE MICRO-SYMBIONTS

Becking (1974) described the family Frankiaceae, with one genus Frankia, including ten species, as the endophytes nodulating the non-legume angiosperms, as follows:

A true mycelium is produced. The mycelium is septate and branched but branching is not necessarily correlated with cross-wall formation.

In an active nitrogen-fixing nodule, the centre of the host cell is filled by a hyphal mass. Near the periphery, spherical or club-shaped terminal swellings are formed on radially arranged hyphae close to the plant cell wall. These spherical bodies are often called vesicles (Fig. 26). The vesicles and club-shaped structures are probably associated with nitrogen-fixation.

No species has as yet been grown on artificial. medium. In previous claims of isolation Koch’s postulates were not fulfilled. The organisms have been grown in root nodule tissue culture, but are often best in subsequent transplants of the tissue because of the difficulty of infecting newly formed callus tissue. The endophyte containing callus tissue does not fix nitrogen.

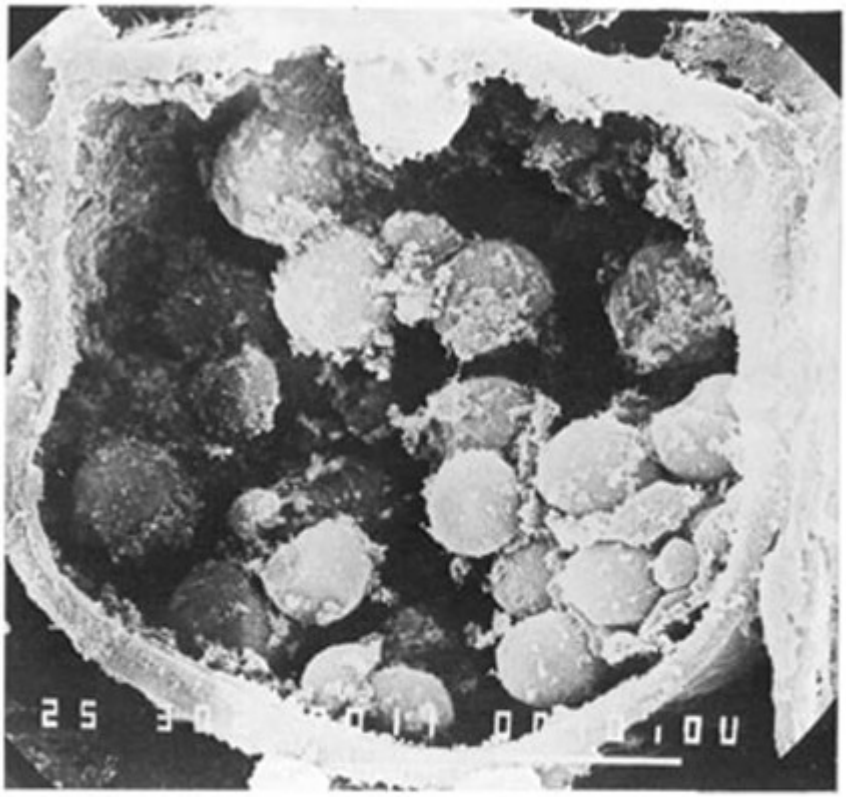


Fig. 26 Infected cell from *Myrica gale* nodules showing endophyte vesicles (x 4 000)
(contributed by J.S. Prent)

The organisms inhabit cortical paranchyme of root nodule tissues as symbionts, but are also present in a free state in soil, presumably as a viable rooting stage, since sterile non-leguminous seedlings nodulate when planted in suitable soil.

The endophyte is specific for a certain plant genus or a group of related genera. In contrast to Rhizobium no exceptions are known; the barriers of incompatibility are strict. It seems appropriate therefore, to give species status to these specific cross-inoculation groups.

Becking (1974) named 10 species of Frankia based on the host they associate with as follows:

Frankia spp	Genera of plants as hosts
F. alnii	Alnus
F. elaeagni	Elaeagnus, Hippophae, Sheperdia
F. discariae	Discaria
F. ceanothi	Ceanothus
F. coriariae	Coriaria
F. dryadis	Dryas
F. purshiae	Purshia
F. cerocarpi	Cerocarpus
F. brunchorstii	Myrica, Gale, Comptonia
F. casuarinae	Casuarina

7.3 CROSS-INOCULATION GROUPS

Like legumes, non-legumes have cross-inoculation groups or barriers of incompatibility (Becking, 1968). Thus a suspension of crushed root nodules of one type will not produce nodulation in another type of non-legume unless they belong to the same cross-inoculation group.

Although no strict barriers of incompatibility are known within any genus, grades of incompatibility exist. Becking (1968) used crushed nodules of Alnus glutinosa (from Wageningen, Netherlands) to inoculate seedlings of A. cordata.(from Europe), A. incana (from Europe), and A. rubra (from California, U.S.A.) raised from sterilized seeds grown in water culture. Table 63 shows that of the Western American species A. rubra, only 28% of the 35 plants in the experimental series were nodulated. In the control series, 100% nodulation occurred; in other species 93 to 96% nodulation occurred.

Table 63 CROSS-INOCULATION IN VARIOUS ALNUS SP (INOCULUM: SUSPENSION OF GROUND A. GLUTINOSA ROOT NODULES, (Becking, 1968)

Species	Percentage nodulated plants[1]
Alnus glutinosa (control)	100.0
A. cordata	95.7
A. incana	92.5
A. rubra	27.7

[1]Values for 35 plants of each species

7.4 NODULES

Root nodules of non-leguminous plants can be divided into two main groups: the Alnus and Myrica-Casuarina types.

Alnus type root nodules are formed in the genus Alnus and other genera of the families Elaeagnaceae, Rhamnaceae, Coriariaceae and in Rosaceae. All these nodules are modified roots with an arrested or very slow growing apical meristem. The nodular lobes are usually dichotomously branched, producing nodules of corallaid appearance (Fig. 27).

Nodules of Casuarina sp and Myrica sp show the characteristic type of nodules in which each root-nodule lobe produces a normal nodule in a negative geotrophic root. Therefore these root nodules become covered with upward growing rootlets.

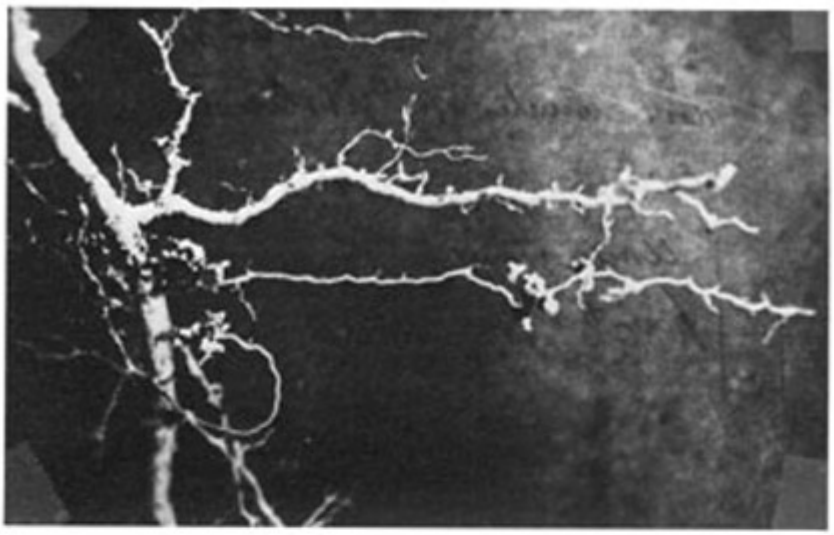


Fig. 27 Types of nodules of *Purshia tridentata* (top) and *Alnus glutinosa* (bottom) (contributed by A.D.L. Akkermans)

Becking (1977) reviewed the processes involved in nodule formation with respect to root hair curling, infection threads, early development of nodules, and their cytology (Fig. 28). Cross sections of the apex of a nodule lobe of alder showed that the endophyte is not distributed arbitrarily in the cortical parenchymat cells of the host. The endophyte is not present in the cortical parenchymatous layers near the stele.

Anatomically, non-leguminous root nodules consist of a central vascular cylinder, surrounded in turn by an endodermis, a cortical parenchymatous layer containing the endophyte and on the outside an epidermal layer. At the tip of the nodule lobe there is an apical meristem.

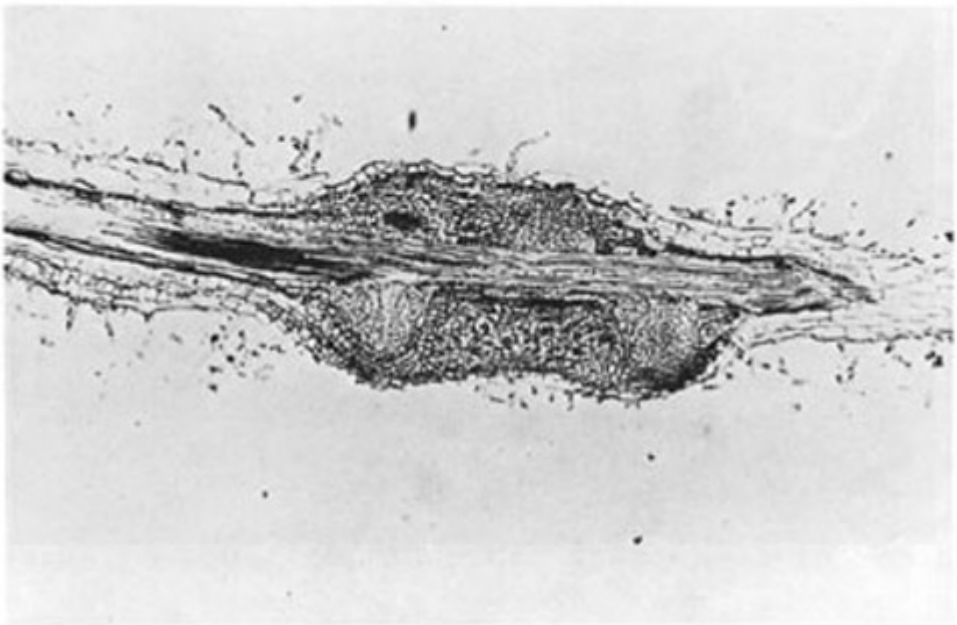


Fig. 28 Primary nodules (2 weeks after inoculation) in cortex and initiation of side vascular bundles. *Alnus glutinosa* inoculated with isolated culture (contributed by A. Quispel)

7.5 EXTENT OF NITROGEN-FIXATION IN SOME NON-LEGUMES

Bond (1963) furnished citations to test the efficiency of nitrogen-fixation of non-legume systems of Alnus, Myrica, Elaeagnus, Hippophae, Shepherdia, Casuarina, Coriaria and Ceanothus. In all cases the results have been equivocal and often dramatic. The nodulated plants grow well, even luxuriantly. In species such as Alnus glutinosa as much as 300 mg nitrogen per plant can be accumulated, by fixation in the first season of growth from seed. In contrast, nodule-free plants, after exhaustion of the seed nitrogen, make little further growth. An example of the amount of nitrogen accumulated per plant is presented in Table 64.

Becking (1977) tabulated the findings, by different authors, on the nitrogen-fixation measurements of some non-legumes (Table 65). Figures ranged from 2 to 300 kg N/ha/year depending on the type and age and location of the plants.

Table 64 ACCUMULATION OF NITROGEN BY NODULATED PLANTS IN ROOTING MEDIA FREE OF COMBINED NITROGEN
(Bond, 1963)

Species	Nitrogen accumulated per plant during first season's growth mg	Subsequent accumulation mg
Alnus glutinosa	300	2500 by end of 2nd season
Myrica gale	146	5020 " " " 3rd "
Hippophae rhamnoides	26	200 " " " 2nd "
Casuarina cunninghamiana	70	1400 after 2.5 "
Elaeagnus angustifolia	-	180 after 1.5 "
Coriaria myrtifolia	36	-

Table 65 FIELD MEASUREMENTS OF NITROGEN-FIXATION IN SOME NON-LEGUMES
(Becking, 1977)

Non-legume	Nitrogen-fixation kg N/ha/Yr.
Casurina equisetifolia	229
Myrica cerifera	3.4
M. rubra (3-15 yr old)	15.25[1]
Alnus crispa (50 yr old)	61.5
A. crispa	40.0
A. crispa (5 yr old)	157.0
A. glutinosa (0-7 yr old)	
(1 plant /m^2)	28.0
(5 plants /m^2)	100.0
A. glutinosa (12 yr old)	26 - 28.0
A. glutinosa	58
A. incana	40
A. rubra	139
A. rubra	up to 300
A. rugosa	193
A. rugosa (natural stand)	85
Hippophae rhamnoides	
(0.3 yr old)	27
(13-16 yr old)	179
H. rhamnoides	
(1-2 yr old)	2
(10-15 yr old)	15
Geanothus spp (natural shrub community)	60
Drys dummondii	18 - 36
D. drummanondii	
Some Shepherdia canadensis	61.5

[1] Data from comparison of Myrica pine stands and pure pine stands. Nitrogen-fixation determined by subtracting total amount N pine stand from total amount N Myrica-pine stand.

Virtanen (1962) demonstrated that the seven-year total content of nitrogen in leaves of an alder (Alnus glutinosa) plant was 14.0 g. From this he calculated that in a grove of alder trees 2.5 m tall with a density of 5 plants/m^2 the total nitrogen gain would be 700 kg/ha.

Lawrence (1958) published results showing that plants associated with sitka alder (Alnus crispa var. sinuata) such as cotton wood (Popadus trichocorpa) weighed 22.5 times as much as those of equal age in a non-alder area. Alder thickets 5 years old and 1.5 m tall added 157 kg/N/ha each year by leaf fall alone.

Dommergues (1963) estimated the increase in nitrogen level of soil from the Cape Verde Islands, off Dakar, West Africa afforested with Casuarina equistifolia. Bare soil contained 80 kg N/ha, but after being under forest for 73 years, the soil yielded 309 kg N/ha i.e. 137 kg N in the Aoo horizon, 28 kg N in the Ao horizon and 144 kg N in the mixed horizon 0-10 cm. In addition the standing timber of the Casuarina forest was estimated to contain 531 kg N/ha. This meant an average net nitrogen-fixation of 58.5 kg N/ha/yr.

Akkermans (1971) showed that an annual nitrogen-fixing activity of approximately 60 kg N/ha/Yr was calculated in alder groves. Since there is a large variation in both the distribution of the nodules and their activity in natural vegetation, higher values up to 130 kg N/ha/Yr may be expected in some places. For Hippophae vegetation a maximum nitrogen-fixing activity of 15 kg N/ha/Yr has been calculated on the basis of the smaller number of nodules produced per unit area.

7.6 OTHER NON-LEGUME NITROGEN-FIXING PLANTS

The nodulation of the plant Trema cannabina var. scabra of the family Ulmaccae Order Urticales was found to be caused by Rhizobium which cross-inoculates with other legumes (Trinick, 1973). Later on this plant was re-examined and identified as Parasponia rugosa BL. (Akkermans et al, 1978).

Nodulation of Zygophyllaceae was also reported to be caused by Rhizobium, with the species Zygophillum album, Z. coccineum, Z. decumbens, Z. simplex, Fagonia arabica and Tribulus alatus (Sabet, 1946; Mustafa and Mahmoud, 1951).

In the nitrogen-fixing species of the genus Gunnera (Order Myrtoles, Family Gunnaraceae) no root nodules are formed, but the symbiont (a blue-green algae Nostoc punctiforme) is an inhabitant of special glands in the stem (Becking, 1977).

The Cycadaceae (Cycads) bear tuberculate roots developed into coralloid formations superficially similar to the nodule clusters of Alnus, Ceanthus etc. The Cycadaceae comprise 9 genera with some 90 species, of which about one third have been recorded to bear such nodule clusters (Bond, 1967).

The nodules of Cycads have blue-green algae of the genera Anabaena or Nostoc inside them. The algae form a deep green zone in the middle cortex of the lobe which is very obvious to the naked eye when a lobe is cut open (Wittman, Bergersen and Kennedy, 1965)

Evidence has been presented (using ^{15}N) that nitrogen-fixation occurs in. the nodules and is followed by a rapid export of fixed nitrogen to other parts of the plants. Bond (1959) showed that Ceratozamia nodules fixed about 1.6% of the total nitrogen of the sample. Bergersen, Kennedy and Wittman (1965) showed that nodules of Macrozamia communis produced 5.2 µg N/g fresh weight/hour.

8. SOME COSTS OF BIOFERTILIZERS

It is generally believed that the immediate gains obtained from using biofertilizers do not compete favourably with the use of inorganic nitrogen fertilizers. However, the long-term benefits, such as increased crops yields together with soil improvement and conservation, increased efficiency of nitrogen utilization, reduced air and water pollution, qualify biofertilizers as partial or complete substitutes for inorganic nitrogen fertilizers.

The cost of production of biofertilizers is relatively low compared to that of mineral fertilizers. Some examples of the cost of legume and algal inoculants is given below.

8.1 LEGUME INOCULANTS

Burton (1972) calculated the cost of inoculating alfalfa seeds in the U.S.A. The cost depends on the method used and whether application is made by the grower or the seedsman. Inoculant for on-farm application costs around U.S. $0.30/ha. The materials for pelleting (inoculant, acacia gum, and calcium bicarbonate) cost about U.S. $1.50/ha. Seeds for 4 ha of alfalfa (50 kg) can be treated in 15 to 30 min. Including labour, the cost of inoculating alfalfa will range from U.S. $0.50/ha for regular inoculation to U.S. $2.50 for seed pelleting.

In India (Subba, Rao, 1972) the cost of 1 000 packets of legume inoculant was given as Rs 600 or 60 paise per packet.

In Egypt, the unit of inoculant is sold to the farmer for U.S. $0.20 (E. £0.15) but the Government subsidises the production costs. From the farmer's point of view costs and savings compared to using mineral fertilizers under Egyptian soil conditions for a crop such as soybean (soils free of R. japonicum) can be calculated as follows:

Without inoculants

Nitrogenous fertilizers required: 60 kg/N/feddan (144 kg/N/ha)
Total cost 60 x E £0.15 = E £9.00 (E £21.6/ha)

With inoculants

Nitrogenous fertilizers required: 15 kg/N/feddan (36 kg/N/ha)
Cost 15 x E £0.15 = E £2.25/feddan (E £5.4/ha)
Inoculant 2 units x E £0.15 = E £0.30/feddan (E £0.72/ha)
Total cost E £2.55/feddan (E £6.1/ha)

Net saving with inoculation: 9.0-2.55 = E £6.45/feddan (E £15.48/ha).

8.2 ALGAL INOCULANTS

Algal technology is being introduced in some rice growing states in India using an open air soil culture method for producing bulk material for field application, through large scale experiment stations, state seed farms, and by village level panchyat unions and individual farmers (Ventkataraman, 1972).

At a state seed farm in Tamil Nadu, algal production costs U.S. $3.65/ha. When chemical fertilizer is used at the rate of 25 kg/ha, it costs U.S. $12.20/ha. Thus using algae, a saving of $9.50/ha is achieved.

Results of using algae in Egypt indicate that $^{1}/_{3}$ to $^{1}/_{2}$ the required mineral fertilizers can be saved. About 60 kg/N/feddan (144 kg/ha) are required for rice cultivation. The cost of this is E £9.00/feddan (E £21.6/ha). With algal application a saving of 3.0 to 4.5 E £/feddan (E £7.2 to 10.8/ha) can be achieved.

METHODS OF ASSESSING BIOLOGICAL NITROGEN-FIXATION

A.l INTRODUCTION

The assessment of the nitrogen-fixing ability of a certain system requires a suitable method to directly detect gains in nitrogen or indirectly the nitrogenase activity. The Kjeldahl method for determination of nitrogen content was introduced in 1883 and was the only available method until the 1940's when the ^{15}N method was adopted to assess nitrogen-fixation in Azotobacter. Subsequently, different methods were developed to ascertain the ability of an organism to fix nitrogen. Table 66 summarizes these methods (Burns and Hardy, 1975).

A.2 KJELDAHL METHOD

In spite of its limitations the Kjeldahl method is still very useful for assessing the response to inoculation, especially in laboratories lacking the specialized equipment required for other methods, although it cannot be used to present conclusive evidence of the capacity of an organism to fix nitrogen. Details on equipment needed, procedure and calculations are presented by Allen (1959) and Vincent (1970). Alternative methods e.g. A value and isotope dilution are also available. (Rennie, Rennie and Fried, 1978. Use of isotopes in biological nitrogen-fixation).

A.3 $^{15}N_2$ INCORPORATION METHOD

The use of stable isotope N_2 was first introduced to confirm nitrogen fixation by azotobacter. The method is 1000-fold more sensitive than the Kjeldahl method and it contributed to a greater understanding of biochemistry and bacteriology of nitrogen-fixation during the 1940's and 1950's.

The method includes:

1) incubation of the test material in the presence of N_2 enriched with ^{15}N.
2) conversion of all N in the sample to ammonia by Kjeldahl digestion.
3) isolation of the ammonia by distillation.
4) oxidation of the ammonia to N_2.
5) mass analysis of N_2 in a mass spectrometer.

Any ^{15}N incorporated by the test material is detected as an increase in the abundance of mass 15 above the background value of 0.364% due to natural abundance of ^{15}N. This increase is expressed as % ^{15}N excess. Samples giving an atom % ^{15}N excess of more than 0.015 could be accepted as positive evidence for N_2 fixation.

A.4 ACETYLENE REDUCTION METHOD

In 1966 it was discovered by Schollhorn and Burris in Wisconsin, and Dilworth in Perth, Australia, that the nitrogenase reduced acetylene to ethylene (Dilworth, 1966). The method based on this principle was soon developed to measure nitrogenase activity in legumes, non-legumes, algae, lake samples, soil samples, etc.

Essentially the method involves incubating the test material in a gas tight container which contains partial pressure of acetylene. At appropriate

Table 66

NITROGEN-FIXATION ASSAYS
(Burns and Hardy, 1975)

Assay method and principle	Nitrogen-fixing Systems		
	Nitrogenase	Organism in culture	Field and natural systems
Growth and morphology:			
Increase of biomass of optical density in N-free medium		++	++
Heterocyst detection (algae) by observation		++	++
Nitrogen based methods:			
Increase in N-content after growth on N_2:			
- Kjeldahl or Dumas		++	++
^{15}N or ^{13}N enrichment in NH_4, Cell components after exposure to $^{15}N_2$ or $^{13}N_2$:			
- mass spectrometry (^{15}N)	++	++	++
- optical mass emission (^{15}N)		+	+
- radioactive counting (^{13}N)	+	+	
Decrease in N_2-Ar ratio following incubation:			
- mass spectrometry		+	
Ammonia formation in extracts following incubation under N_2:			
- titrimetry	++		
- calorimetry	++		
N_2, H_2 uptake by extracts:			
- manometry	+		
Methods based on ability of nitrogenase to reduce substrates other than N_2:			
C_2H_2 reduction to C_2H_4:			
- gas chromatography	++	++	++
- calorimetry			
Nitrile, isonitrile reduction:			
- gas chromatography	+	+	
HO_3 reduction to H_2:			
- manometry	++		
- gas chromatography	+		
- mass spectrometry	+		
Methods based on utilization of dithionite:			
- UV spectrophotometry	+		

+ indicates at least one report.

++ indicates frequent use.

times samples of the atmosphere may be withdrawn by syringe for immediate or eventual analysis by using flame ionization after gas chromatography. The amount of C_2H_4 detected is correlated with the intensity of nitrogenase activity in the sample. Values for nitrogenase obtained by this method are conventionally designated as N_2 (C_2H_2) fixing activity and nitrogenase (C_2H_2) activity.

Burns and Hardy (1975) evaluate the method as follows:

i. Sensitivity: It is about 10^3 times more sensitive than $^{15}N_2$.

ii Facility: Because both C_2H_4 are gases the application of substrate C_2H_2 and recovery of product C_2H_4 require only simple gas handling techniques. The gas chromatograph and incubation equipment are likewise simple to use.

iii. Internal standard: C_2H_2 is a natural internal standard for the assay and it is measured readily in conjunction with the product C_2H_4.

iv. Specificity: Product C_2H_4 is readily separated and quantified on the gas chromatograph.

v. Rapidity: About 80 incubations and about 160 gas chromatographic analyses per man-day are feasible.

vi. Product stability: The gas samples containing product C_2H_4 can be stored indefinitely in gas-tight containers for chromatographic analyses.

vii. Economy and mobility: All apparatus used is relatively inexpensive and rugged enough for use in all terrain-type mobile units.

viii. Sample conservation: The test material need not be sacrificed for analysis, and normally can be repeatedly sampled because each analysis requires only a small fraction of the incubation atmosphere.

The disadvantages of the method include the explosive nature of C_2H_2 and the indirect nature of the reaction in the sense that C_2H_2 is not the physiological substrate. In most studies the correlation between C_2H_2 reduced and N_2 reduced indicates that the reduction of 3 to 4 mol C_2H_2 reflects nitrogenase activity capable of reducing one mole of N_2. This relationship is consistent with the amount of reductant needed, since C_2H_2 reduction requires 2 e^-, and N_2 reduction requires 6 e^-. However, variability in the conversion factor has been observed, and the most reliable data for aiven conditions are obtained by establishing a factor for each particular variation in experimental procedure.

Sample Preparation

Assays should be made immediately after sampling (within 2 hours). Nodules are best assayed while still attached to the roots. Removing the tops has no measurable effect on short term assays.

Some N_2-fixing systems are apparently cold labile and therefore samples should not be stored cold before assay. For comparisons, sampling and assays should take place at the same time each day because the nitrogenase activity of nodulated roots may vary with the light intensity (photosynthetic activity) and temperature throughout the day. When possible, nodulated roots should be shaken free from soil and assayed without washing. Activity is diminished when the surface film of water remaining after washing nodulated roots is not carefully removed and drying of nodules also diminishes activity. Samples are assayed in conveniently sized containers, e.g. 50 ml syringes or glass bottles, with a rubber septum in the stopper to allow it to be pierced by a hypodermic needle.

The activity of nodules is decreased as the partial oxygen pressure (pO_2) falls below 0.20 atm. For rhizosphere, non-symbiotic organisms, a pO_2 of about 0.04 atm is often optimal. Assay vessels should be large enough to minimize change in pO_2. Liquid samples should be well shaken during assay. It is preferable to assay soil as intact cores. Samples containing blue-green algae should be illuminated at light intensities corresponding to those in situ in the field.

For assays of soil-plant systems, soil cores can be placed in an air tight plastic container (such as a plastic plant pot with a clear plastic bag of rigid, clear plastic cover) connected to the base by a water seal. The containers should be incubated in the light as the activity of rhizosphere-associated nitrogen-fixing bacteria is influenced by photosynthesis. A known amount of propane to give a concentration of 100 ppm is injected and used as an internal calibration standard to measure the volume of gas in the incubation chamber and to monitor leaks and other losses of C_2H_4.

Assay Procedure

For active systems e.g. nodules or blue-green algae, assays can be made in air, provided a concentration of 10% C_2H_2 is used. The atmosphere in the vials can be altered by alternately evacuating to 30 mm Hg and flushing with an appropriate gas mixture (usually Ar/O_2) two or three times.

Some gas withdrawn from the sample container by syringe and replaced with acetylene to give a final concentration of 10% of acetylene may be premixed with the gas phase introduced into the sample container. Samples without C_2H_2 added should be assayed for C_2H_2 produced endogenously. Samples are best incubated at temperatures which are either constant or related to the in vivo temperature.

After a suitable time (30 min for legume root nodules, up to 24 hours for soil) gas samples 0.5-2 ml are taken with a syringe. For periods up to about 3 hours, the gas sample can be stored by sticking the syringe needle into a rubber bung. For storing gas samples over longer periods, samples can be transferred into pre-evacuated containers.

The gas colume of the assay vessel is measured by displacement with water (using a burette) after assay. This is not necessary with propane used as an internal standard.

Gas Chromatography

Gas mixtures can be separated by gas chromatography and then quantified by flame ionization or thermal conductivity detectors. For acetylene, ethylene and propane, various column packing materials can be used. A convenient system to use is 80-100 mesh porapak N or T in a 2m x 0.003m diameter, stainless steel column at 100 °C, with a nitrogen gas flow rate of 25 ml/min using a hydrogen/air flame ionization detector. Another convenient system is 100:0 NO_3 PO_4 on spherosil x 0.3 cm glass column at 35°C with a nitrogen carrier gas flow rate of 4 ml/min. Most detectors can routinely detect 0.1 ppm C_2H_2 in a 0.5 ml gas sample. For porapak N or T, CH_4 (propane) has the shortest retention time followed by C_2H_4 (ethylene), C_2H_2 (acetylene) and C_3H_8 (acetone). For a Na_3PO_4 column, however, propane is eluted before the acetylene. At most concentrations encountered in the assay, peak heights can be taken linearly and be related to the concentration with fair accuracy.

Calculation

A suitable calculation for ethylene (C_2H_4) produced in moles C_2H_4/h is as follows:

$$(C_2H_4 \text{ sample CU} \times \frac{\text{Vol gas in sample container}}{\text{Vol injected into GLC}} \times \text{assay time (h)} \times K)$$

$$- (C_2H_4 \text{ blank CU} \times \frac{\text{Vol gas in blank container}}{\text{Vol injected into GLC}} \times K)$$

where: CU = Chart units used to measure peak height.
blank = Sample container with added C_2H_2 only.
K = Conversion factor obtained using a standard C_2H_4 gas mixture to calibrate the chromatograph.

For example, for 100 ppm C_2H_2 standard, K is derived as follows:

1 ml of 100 ppm C_2H_2 contains

100×10^{-6} ml C_2H_4 and = X CU

22.4 C_2H_4 at STP = 1 mole C_2H_4

$$1 \text{ ml of } 100 \text{ ppm } C_2H_4 = \frac{100 \times 10^{-6}}{22.4 \times 10^3} \text{ moles } C_2H_4$$

= 0.00446 m moles C_2H_4 = X CU

Then K (or CU) = $\frac{0.00446 \text{ m moles } C_2H_4}{X}$

Precautions and Comments

i. C_2H_4 is soluble in silicon rubber and autoclaving produces C_2H_4 for rubber such as subaseals. C_2H_4 contamination in rubber can be decreased by steaming with water for 10-15 minutes and leaving to stand 24 hours before further use.

ii. Disposable plastic syringes and 25 g needles (0.5 mm diameter) are suitable for samples and gas transfers. Contaminating C_2H_4 can be decreased by evacuating and flushing the syringes with air about 6 times.

iii. C_2H_2 can be bought in cylinders (Welding grade is suitable) or it can be produced by reacting calcium carbide with water. C_2H_2 from either source contains variable amounts of contaminating C_2H_4 (in addition to CH_4 and phosphine) and suitable control blank samples should be taken to monitor this.

iv. C_2H_2 can be used as an internal standard in the assay to monitor sampling and injection errors. Propane can also be used in the assay atmosphere as a standard, specially for assays of soil-plant systems.

v. The chromatography of each set of assays should be calibrated. A standard, premixed cylinder of C_2H_4 (C. 100 ppm) in Ar or N_2 is useful for this.

vi. C_2H_4 is a plant growth hormone, and most responses are saturated by 2 ppm C_2H_4. Plant membrane integrity is affected by C_2H_4 and/or C_2H_2.

vii. Because C_2H_2 is more soluble than N_2 in water, the theoretical conversion factor of 3/2 may not apply and the actual ratio should be found by using ^{15}N or Kjeldahl methods.

RHIZOBIA

B.l. RECOGNITION AND CHARACTERIZATION OF RHIZOBIUM

The decision as to whether a culture is or is not a rhizobium usually depends on a plant test. Certain conformable and contradictory characteristics are worth noting (Vincent, 1970).

Some distinction between groups of Rhizobium and the recognition of Agrobacterium is likely to be possible on the basis of cultural and biochemical characteristics (Graham and Parker, 1964). The general characteristics are outlined in Table 67. Typical R. japonicum - R. lupini-Cowpea rhizobia produce very small colonies and utilize citrate poorly. They commonly produce penicillinase, and do not cause a presipitate in Ca glycerol phosphate medium.

Plant tests that permit reasonable distinction between present species (Graham and Parker, 1964) are presented in Table 68. On some occasions strains of R. phaseoli could be expected to show rather more cross reactivity with other hosts of the clover-pea-vetch-bean group. However, the three species, or symbionts, in this group can be generally distinguished by their preferred host where the reaction often results in a much more effective association than in other seemingly less compatible cases. Similarly, among the slow growers, separation into the present subgroups is indicated by the nature of the preferred host. R. meliloti can almost always be readily distinguished from the other rhizobia, as well as from the agrobacteria by its nodulation of Medicago sativa.

Table 67 CULTURAL AND BIOCHEMICAL CHARACTERISTICS OF MAJOR GROUPS OF RHIZOBIA AND DISTINCTION FROM AGROBACTERIUM
(Collected from Graham and Parker, 1963).

	R. trifolii R. leguminosarum R. phaseoli	R. meliloti	R. japonicum R. lupini Cowpea rhizobia	Agrobacterium
i. Colony size, mm	2+	2+	1	2+
ii. Raffinose used	0.84	0.73	0.19	0.89
iii. Citrate used	0.03	0.27	0	0.72
iv. Growth pH 4.5	0.65	0.09	0.83	0.88
v. Growth pH 9.5	0	0.91	0	0.56
vi. Growth 2% NaCl	0	0.45	0	0.61
vii Growth 39°C	0	0.72	0	0.06
viii.Response to thiamine	0.65	0	0	0.11
ix. Response to pantothenate	0.89	0	0.03	0
x. Response to biotin	0.59	0.36	0.23	0
xi. H_2S produced	0	0.81	0	1.0
xii. Pénicillinase produced	0.08	0.08	0.77	0.11
xiii.Precipitate from Ca glycerophosphate	0	0.55	0	1.0

Entries, except in the case of i. represent the proportion of rhizobia strains in each group, or species, showing the characteristics.

Table 68 Nodulation specificity of Rhizobial species
(Collected from Graham and Parker, 1964).

Test host	R.legum-inosarum	R.trifo-lii	R.phase-oli	R.lup-ini	R.japo-nicum	Cowpea rhizo-bia	R.meli-loti
Vicia sativa	0.85	0.1	0	0	0	0	0
Trifolium repense	0.4	0.7[2]	0	0	0	0	0
Phaseolus vulgaris	0.15	0.05	1.0	0.2	0.7	0.5	0
Macroptillium pathyroides[3]	0	0	0.8	0	0.3	0.2	0
Ornithopus sativus	0	0	0	1.0	0.3	0.05	0
Glycine max	0	0	0	0.1	1.0	0.3	0
Vigna sinensis	0	0	0	0.2	0.3	0.5	0
Medicago sativa [4]	0	0	0	0	0	0	1.0

1/ Entries represent the proportion of rhizobial strains in each species nodulating the host.
2/ Partly reflects poor compatibility of isolates from Agrican clovers; those from T.ambiguum are poorly compatible with Mediterranean clovers.
3/ Formerly Phaseolus: M.atropurpureum is widely susceptible to slow growing rhizobia including isolates from Lotoraonis and Leucaena which are host specific.
4/ Whereas M.sativa appears to be always invasible by R.meliloti, other species of Medicago are likely to be more strain specific. Some strains from Leucaena nodulate M.sativa.

B.2. LITMUS MILK REACTION

Reactions in litmus milk (after 1-6 weeks) have been used to record changes in the acid or alkaline direction, and the development of a digested “serum” zone which is a feature of some strains of Rhizobium. Non-rhizobial forms or contamination will often be revealed by their rapid growth in this medium, frequently accompanied by rapid change of reaction and decolouriza-tion of litmus.

In general, litmus milk reactions provide more reliable evidence for purity of rhizobia than for identification of species. With the exception of R.meliloti, fast growing strains tend to produce an alkaline reaction in litmus milk accompanied by the formation of a wide serum zone. An acid reaction and acid serum zone in litmus milk are produced by strains of R.meliloti. No serum zone accompanies the alkaline reaction brought about by members of slow growing rhizobia as exemplified by R.lupini, R.japonicum, cowpea rhizobia and related forms. None of the rhizobia peptonize or coagulate milk. All reactions in litmus milk are produced slowly and are best judged after several weeks incubation at 25-30°C.

B.3. TECHNIQUES APPLIED IN THE STUDY OF RHIZOBIAL ECOLOGY

i. Selective media for rhizobia

A medium which selects rhizobia and inhibits other microorganisms in soil has not yet been formulated. Attempts have been made to develop media to select rhizobia from soil but none have been satisfactory.

ii. Plant dilution (plant infection) techniques

The selective media most widely used to detect rhizobia in soil has been the legume host itself. The method depends on the eventual appearance of a nodule on the roots of a legume test plant as the indicator of an appropriate Rhizobium in the soil. Numbers of rhizobia are usually estimated by preparing 10 fold dilutions as inocula for replicate rhizobia-free seedlings. This "plant dilution" technique is thus a variation of the statistical most probable number (MPN) assay and based on the assumption that a single Rhizobium can initiate nodulation.

iii. Serological methods

Serology has been used in ecological studies of rhizobia primarily to identify different strains of rhizobia as to:

a. Survival of inoculant strain.
b. Competition among inoculant strains, and
c. Nature of indigenous soil rhizobia.

The test is usually formed by exposing the systems to a legume host plant, isolating rhizobia from the nodules that are formed, and studying the occurrence of the serotype(s) among the nodule isolates. Agglutination technique is widely used. Immuno diffusion technique has also been applied (Vincent, 1970).

iv. Immunological approaches

The fluorescent antibody (FA) technique is a general one for ecological study of microorganisms in natural environments. Antibodies against the micro-organisms to be studied are prepared and after the addition of a fluorochrome, the antibody is used as a highly selective stain for the direct microscopic examination of the natural environment. The antigen-antibody reaction is visualized by fluorescence microscopy to allow for the simultaneous detection and recognition of the microorganisms of interest. The technique is now developed to detect and recognize specific strains of rhizobia in soil and to observe some qualitative interrelationships of that strain with its environment. To use the FA to quantify rhizobia in soil, population should not be less than 10^3 to 10^4 depending on the properties of the soil.

v. Mutant markers and phage typing

Mutants resistant to the antibiotic streptomycin were first proposed by Obaton (1971). Streptomycin resistant mutations are readily obtained spontaneously at adequately high level in a single step and are stable. Rhizobia so marked must first be tested to ensure that the mutant has not lost symbiotic effectiveness, as some strains may loose efficiency.

After inoculation of soil or seed the mutant may be identified by recovery from nodules plated on appropriate media containing antibiotic. Other antibiotics e.g. spectinomycin, can be used alone or with streptomycin as double markers.

Phage typing is used as a means of distinguishing among closely related strains of rhizobia. Rhizobiophage commonly occurs in nodules and field soils cropped to legumes.

B.4. LEGUME SEED PELLETING

Lime pelleting of legume seeds is becoming a commonplace operation on the farm. Its main purpose is to protect the root nodule bacteria from harmful conditions and thereby produce an improved environment for their survival on the seed. Several advantages are obtained by lime pelleting (Roughley, Date and Walker, 1965) as follows:

it counteracts soil or fertilizer acidity, or both, in the micro-environment of the seed, allowing improved survival of the added rhizobia:

it makes aerial sowing of inoculated seed a practical proposition:

it ensures better survival of the bacteria when days between inoculation (pelleting) and sowing are unavoidable:

it improves the chance of obtaining promptly nodulated plants by ensuring better survival of rhizobia after sowing especially when germination is delayed.

Adhesive. A high grade gum arabic or gum acacia is the best adhesive agent. It is essential that the gum is free from preservatives. Very finely ground gum is hard to dissolve in water (8-mesh ground dissolves satisfactorily). Other adhesives are methofas R, cellofas A, and methocel R. These do not provide the same degree of protection for the rhizobia as does gum arabic.

Pelleting material. A finely ground form of calcium carbonate that will pass at least 300 mesh seive is required.

The inoculum. Peat inoculants are preferred or other satisfactory inoculants can be used.

Equipment. Small batches of seed (up to 5 lb) can be pelleted satisfactorily by hand, in a dish. Larger quantities can be pelleted very satisfactorily in a clean cement mixer or revolving drum.

In the absence of these facilities, reasonable pellets have been made by simply rolling the ingredients in a canvas sheet by alternately lifting one end toward the other until smooth pellets are formed.

Preparing the pellets

A summary of the steps to be taken and rates of materials recommended for pelleting seed are given below.

Ingredient	Quantity	Operation
Gum arabic	3.5 fl oz (99 ml)	Dissolve gum arabic in water.
Drinking water	8 fl oz (227 ml)	This makes ½ pint (0.28 1) of gum solution.
Inoculum	1 packet	Add inoculum to gum solution and mix thoroughly.
Seed:		
- small, e.g. white clover	15 lb (6.8 kg)	Add the gum inoculum mixture to the
- medium, e.g. sub clover	30 lb (13.6 kg)	seed, and mix until seed is evenly
- large, e.g. vetch	60 lb (27.3 kg)	coated.
Fine lime	7½ lb (3.4 kg)	Add the lime all at once and mix rapidly for 1 to 1½ min only.

Characteristics of a good pellet

- The seed should be evenly coated by the lime.
- After the mixing, the pellet should appear dry but without loose lime left on the surface giving it a powdery look.
- Free lime should not be left in the mixer.
- Pellets should be firm enough to drop on the floor without damage.
- When dry, the pellets should withstand light rolling between the fingers.

B.5. TECHNIQUES FOR QUALITY CONTROL OF LEGUME INOCULANTS
(Roughley, personnel contact)

Quality control must begin with the selection and maintenance of mother culture, and include control of the broth before addition to the carrier and the finished product. All broths must be numbered and the batch of inoculant prepared from them given the same number or numbers. Each container of inoculum should also carry this number so that if there is a nodulation failure, the source of the inoculum can be traced.

CONTROL OF MOTHER CULTURES

i. Each year strains used for commercial production should be streaked onto yeast extract mannitol agar.

ii. If pure, subculture onto YMA in a screw-capped bottle or test tube.

iii Check the resulting growth by:
   a. Gram stain
   b. Growth on glucose-peptone agar
   c. Check by agglutination that the culture is true to type

iv. Subculture onto enough screw-capped tubes to supply mother cultures for 1 year to reduce subculturing strains to a minimum.

v. Inoculate 12 plants growing in N-free agar in tubes with a loop of culture taken from each of the tubes prepared in iv. Compare growth with uninoculated control plants.

CONTROL OF BROTH CULTURES

Each broth must be tested before impregnating the carrier. Because some tests take more than 2 days, the peat will be impregnated before all the results are known. Should the results of these later tests fail to meet the minimum standard the inoculant must be discarded.

<u>Preliminary Tests Which Must be Completed Before the Carrier is Impregnated</u>:

i. Streak broth into glucose peptone brom-cresol purple agar in duplicate tubes. Incubate at 30°C. Examine f or growth and whether or not acid or alkali was produced after 24 hours. Most rhizobia fail to grow on this media and usually do not produce acid (yellow). Some strains make a little growth after 48 hours and may turn the media alkaline (purple).

ii. Gram-stain: rhizobia are gram negative. Reject any broth with contaminants present.

iii. Total cell count: broth must contain at least 1000 x 10^6 cells/ml. If fewer, reject the broth.

iv. Serological check: to verify that the correct strain is present, mix 3 ml of broth with 3 ml of 0.85% saline in a test tube and boil for $^1/_2$ hour (to remove "H" antigen).

Test by using 3 serology tubes:

a. 2 for test
b. 1 for control

Use a pasteur pipette to add to an agglutination tube:

a. 18 drops of boiled broth-saline plus 2 drops of antisera, and
b. 18 drops of boiled broth-saline plus 2 drops of 0.85% saline.

Incubate in water bath at 45° C for 4-5 hours.

Result: Agglutination of cells compared with control idicates a positive result.

Tests Which Require More Time for the Results

Total viable count. This is an extremely important test: To make satisfactory inoculants a broth must contain at least 500 x 10^6 viable rhizobia/ml. If there are fewer, the impregnated carrier must be discarded.

Dilute the broth in sterile tap water to a final concentration of 10^{-8}. Plate out aliquots at 10^{-6}, 10^{-7} and 10^{-8} onto duplicate plates of YMA. Incubate at 26°C. Count the most suitable dilution after the appropriate time.

CONTROL OF THE FINAL INOCULANT

At least five containers from each batch should be subjected to the tests detailed below. If more than one fails, reject the batch. If one only fails test a further 5 containers. Should any of the second set of 5 fail reject the batch.

Viable Counts of Rhizobia by Plate and Most Probable Number Count (MPN) Methods

i. Add 30 g carrier to 270 ml sterile water and shake for 10-15 minutes (10 g carrier to 90 ml sterile water may be used as an alternative i.e. 10^{-1}dilution).

ii. Prepare further dilutions of 10^{-6},10^{-7},10^{-8},10^{-9},10^{-10}

iii. Plate out 1 ml aliquots from the 10^{-6} and 10^{-7} dilutions onto YMA + Congo red. Incubate at 26°C for appropriate time.

iv. Transfer 1 ml from dilutions 10^{-7},10^{-8},10^{-9},10^{-10} into each of 2 tubes with a sterilized seedling of the appropriate host growing in N-free agar. Examine for nodulation after 4-6 weeks and calculate the number of rhizobia in the inoculant from the most probable number tables.

Cultures in unsterilized carriers should contain a minimum of 500 x 10^6 rhizobia/g carrier. In sterilized carriers the minimum number should be 1000 x 10^6/g.

CULTURE MEDIA UTILIZED FOR RHIZOBIA

Medium for Use in Fermentation

K_2HPO_4	0.75 g
$MgSO_4.7H_2O$	0.4 g
$CaCO_3$	0.4 g
Yeast extract[1]	3.0 g
Sucrose	10.0 g
Water	1 litre
pH	6.8

[1]/ Commercial yeast extract preparation. Alternatively yeast extract may he prepared using compressed yeast (described under yeast extract mannitol agar recipe). In this case add 100 ml of extract and reduce the water to 900 ml.

Yeast Extract Mannitol Agar with Congo Red Indicator for Plate Counts of Broth and Inoculants

Constituents

Yeast extract	3.0 gm	(dehydrated) or 100.0 ml of yeast extract
$MgSO_4.7H_2O$	0.2	solution.
K_2HPO_4	0.5	
Mannitol	10.0	
Agar	15.0	
Congo red (1:400 aqueous solution)	10.0 ml	
Water (pH 6.8)	1 000.0 ml	

Preparation of Yeast Extract Solution

Constituents

Compressed yeast	400.0 gm
Deionised water	4.0 litres

Method

i. Add yeast to water and let stand for 2 hours
ii. Autoclave for 3 mins
iii. Stand for 2 days
iv. Decant clear liquid and dispense into flasks
v. Autoclave for 30 mins

To use: 100 ml/litre media

Glucose Peptone Agar

Constituents

Glucose	5.0 gm
Bacteriological peptone	10.0 gm
Agar	15.0 gm
Water	1 litre

1.6% alcoholic brom-cresol purple

Method of preparation:

i. Add peptone and agar to water. Autoclave at 10 lb/in^2 for 5 minutes.

ii. Add glucose, brom-cresol purple, dispense in tubes and autoclave at 10 lb /in^2 for 10 minutes.

N.B. Do not autoclave glucose twice.

Plant Medium (Nitrogen Free) Used for Tests of Effectiveness and Counting Rhizobia (MPN)

Constituents

$CaHPO_4$	1.0 gm
K_2HPO_4	0.2
$MgSO_4 . 7H_2O$	0.2
NaCl	0.2
FeCl	0.1
Agar	15.0
Water	1 litre

Other salts added as required e.g. trace elements

B.6. METHOD FOR SEED STERILIZATION FOR PLANTS USED IN COUNTING RHIZOBIA. (MPN)

Mercuric Chloride Sterilization

i. Wash seed with alcohol for ½ minute.

ii. Pour off alcohol and wash in mercuric chloride for 2½ minutes.

iii. Pour off mercuric chloride and wash seeds with sterile water six quick washes then leave seeds soaking in water for several hours.

iv. Spread seeds thinly in petri dishes containing plain agar. Incubate.

Mercuric chloride ($HgCl_2$) - for seed sterilization

$HgCl_2$	1.0 gm
Conc. HCl	5.0 ml
Distilled water	500.0 ml

Seeds which may be sterilized with mercuric chloride

Clovers-sub, white, rose, red, strawberry

Medics - lucerne, paragosa

Vetch - for good germination, leave seeds soaking in water, after sterilization overnight.

Lotus bean

Seed may have to be washed free of fungicide before sterilizing. After sterilizing, place seed in closed container on damp cotton wool until germinated. This may take 4-5 days.

Acid Sterilization - Using Concentrated Sulphuric Acid

i. Wash seeds with acid for 10 minutes

ii . Pour off acid carefully, then wash seeds quickly in six washes of sterile water. Leave seeds soaking for several hours.

iii. Spread seeds thinly in petri dishes containing plan agar. Incubate.

Seeds which may be sterilized with concentrated sulphuric acid

Lotononis sp

Siratro

Desmodium

Fine stem stylo

Trifolium semipilosum

Soak in acid for 20 minutes then proceed as described above.

FREE-LIVING NITROGEN FIXERS: *Azotobacter, Beijerinckia, spirillum*

Different media are developed for detecting, isolating and counting free-living nitrogen fixers. The following is a description of different media commonly used.

Modified Ashbys' medium (Hegazi and Niemela, 1976) is usually used for isolating and counting of *Azotobacter* sp.

	g/l
Glucose	20.0
K_2HPO_4	0.5
$MgSO_4.7H_2O$	0.2
NaCl	0.2
$CaSO_4.2H_2O$	0.1
$CaCO_3$	5.0
$MnSO_4.4H_2O$	0.002
$NaMO_4.2H_2O$	0.002
Agar	20.0

For the most probable number counts, the following medium is generally recommended (Abdel-Malek and Ishac, 1968).

	g/l
Mannitol	10.0
Sucrose	10.0
K_2HPO_4	0.5
$MgSO_4.7H_2O$	0.2
NaCl	0.2
Agar	1.5
$CaCO_3$	5.0
Traces of, M, SO_4, $4H_2O$	
Ferrous sulphate	
Molybdic acid	

A. paspali is counted by using the following medium (Dobereiner, Day and Dart, 1972). Growth of *A. paspali* on plates incubated at 35°C appeared after 2 days incubation as round, raised dense colonies which turned yellow because of dye absorption and acid production.

K_2HPO_4	0.005%
KH_2PO_4	0.015%
$MgSO_4.7H_2O$	0.02%
$FeCl_3$	0.001%
$NaMoO_4.2H_2O$	0.0002%
Sucrose	2.0%
Bromthymol blue	5 ml/l (of 1% alcohol sol.)
pH	6.9 $\pm$ 0.1

Isolation media for *Beijerinckia* (Becking 1961).

The selective enrichment medium is of the following composition.

	g/l
Glucose	20
KH_2PO_4	1.0
$MgSO_4.7H_2O$	0.5
Distilled water	1000
pH	5

Medium for pure culture.

Glucose	20.0
KH_2PO_4	1.0
$MgSO_4$	0.5
$FeCl_3.6H_2O$	0.1
$NaMO_4$	0.02
Agar	20.0

A medium to distinguish the bacteria of *Derxia* sp was developed by Dobereiner and Campelo, (1971). *Derxia* colonies appear as raised, glistening and red brown, smooth with tough rubbery consistency.

	g/l
Starch	20
K_2HPO_4	0.05
KH_2PO_4	0.15
$MgSO_4.7H_2O$	0.20
$CaCl_2$	0.02
$FeCl_3$	0.01
$NaMoO_4.2H_2O$	0.002
bromothymol blue (0.5% in ethanol)	5 ml
$NaHCO_3$	1.0
Agar	20

Media for the isolation and growing of *Azospirillum* sp.

Dobereiner *et al* (1972b) medium for enrichment:

K_2HPO_4	0.4 g
KH_2PO_4	0.1 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.1 g
$CaCl_2$	20.0 mg
$FeCl_3$	10.0 mg
$NaMoO_4.2H_2O$	2.0 mg
Agar	1.75 g
H_2O	1000 ml

Dobereiner *et al* (1976) medium for growth.

	g/l
Malic acid	5.0
KH_2PO_4	0.4
$MgSO_4.7H_2O$	0.2
NaCl	0.1
$CaCl_2.7H_2O$	0.02
$FeCl_3.6H_2O$	0.01
$NaMoO_4.2H_2O$	0.002
B. thymol blue (0.5% aqueous)	2 ml
KOH	4.0
Agar	1.75
Water	1000 ml
pH	6.8

Okon *et al* (1977) medium

K_2HPO_4	6.0 g) mixed in 10% of the final 4.0 g) volume and autoclaved separately and added to the cold medium.
$MgSO_4.7H_2O$	0.2 g
NaCl	0.1 g
$CaCl_2$	0.02 g
Malic acid	5.0 g
NaOH	3.0 g
Yeast extract	0.1 g
NH_4Cl	1.0
$FeCl_3$	10.0 mg
$NaMoO_4.2H_2O$	2.0 mg
$MnSO_4$	2.1 mg
H_3BO_3	2.8 mg
$Cu(No_3)_2.3H_2O$	0.04 mg
$ZnSO_4.7H_2O$	0.24 mg
pH	6.8

BLUE-GREEN ALGAE

D.1. ^{15}N TRACER

Williams and Burris (1952) developed a method for testing Calothrix parietina as follows.

After the algal cultures have used most of the fixed nitrogen, they are concentrated aseptically by decantation or centrifugation and are resuspended in 100 ml of fresh Chu 10 medium containing one mg fixed nitrogen per ml. The medium is modified by substituting $CaCl_2$ for $Ca(NO_3)_2$ and the nitrogen is supplied as NH_4NO_3. The 250 ml bottles are closed with sterile rubber stoppers carrying cotton plugged inlet tubes.

The bottles are evacuated and assed with 0.25 atom ^{15}N-enriched N_2, 0.50 atom argon, 0.20 atom O_2 and 0.05 atom CO_2. These cultures are illuminated and remain stagnant. They are not opened until time for harvest (4-30 days). At harvest, cells are sedimented, resuspended in distilled water and washed by centrifugation.

The cells are subjected to Kjeldahl digestion, the ammonia is distilled and then converted to N_2 with alkaline hypobromide. The N_2 is analysed for ^{15}N with a mass spectrometer.

By this method, 0.003 atom per cent of ^{15}N excess can be detected and 0.015 atom per cent ^{15}N excess will serve as a conservative level for establishing fixation of ^{15}N.

D.2. DETERMINATION OF AMMONIA IN CULTURES OF BLUE-GREEN ALGAE

(Solorzano, 1969)

Reagents

i. Phenol alcohol solution, 10 g phenol in 100 ml of 95% C_2H_5OH.

ii. Sodium nitoprusside solution, 0.5% W/V in water.

iii. Alkaline hypochlorite solution. This is prepared by mixing two solutions in a ratio of 4 vols of a: 1 vol of b.

a. 100 g of trisodium citrate + 5 g NaOH in 500 ml H_2O.

b. Sodium hypochlorite solution.

Reagent i will keep well for some months.

Reagent ii will keep for one month if kept in brown bottles.

Reagent iii should be prepared daily.

Solution a will keep for some months as well as b if stored in a refridgerator (40°C).

Procedure

Standard curve: using a range of concentrations of NH_4Cl in distilled water, pipette 8 ml of solution into triplicate test tubes (range from 0 to 1 mmol). Add 0.2 ml of phenol alcohol solution, followed by 0.2 ml of sodium nitropruside solution followed by 0.5 ml of alkaline hypochlorite solution. Shake well between each addition. Allow to stand at room temperature (22-27°C) for 1 hour and read absorbence at 640 nm using a reagent blank as zero. The colour will remain unchanged over 24 hours. Glassware should be acid washed before use.

Samples

Use 5 ml (or smaller volume made up to 5 ml with distilled water) of supernatant prepared by centrifuging or filtering the cultures. Add reagents and treat as for standard curve. Read values of ammonia concentration from standard curve.

D.3. IN SITU ASSAY OF NITROGEN FIXING ACTIVITY

An in situ acetylene reduction assay technique for use in paddy fields, using plastic bags and bottomless metal frames has been developed. The plastic bag chamber is convenient for addition and removal of gas. This technique has been used by Watanabe et al (1977) to estimate nitrogen fixation in rice fields.

D.4. NITROGENASE (C_2H_2) ACTIVITY OF BLUE-GREEN ALGAE

As an example (El-Sayed, 1978). 25 ml samples of algal suspension were transferred into 420 ml capacity serum bottles. The bottles were then sealed with tight silicon rubber subseals. The suitable volume of acetylene was injected into the bottles to satisfy an acetylene concentration of 10% as recommended, without removing air, and the pressure was then restored to atmospheric by pricking the seals with a syringe needle. The bottles were incubated under illumination at 30°C for 2 hr after which samples of gas phase were withdrawn into a syringe and stored until subsequent analysis for ethylene production. Two ml of gas samples were injected into a Pye Unicum GLC Model 104, fitted with 183 cm long and 0.4 cm diam stainless steel column, filled with 10% NO_3PO_4 on 100-120 mesh Spherosil X OB and with dual flame ionization detection. The carrier gas was nitrogen at a flow rate of 28 ml/min, hydrogen and air at rates of 30 and 300 ml/min, respectively. Temperatures were 35°C for column, 60°C for injection and 150°C for detector.

During analysis 0.5-2 ml of standard gas mixture (air and acetylene 9:1 containing 100 ppm ethylene gas) were injected.

To calculate the amount of ethylene produced by the samples, the heights of the samples and standard peaks were compared. Results were presented as namomol C_2H_4/mg dry wt/h.

D.5. SILICA GEL PLATES

Silica gel plates are used in one or more steps for isolating and purifying blue-green algae. To each petri dish 0.2 g of powdered $CaCO_3$ is added followed by the addition of 20 ml of sodium silicate solution 8.5%. Acid solution (composed of 20 ml of liquid modified Chu 10 medium, 300 ml of HCL 30% v/v and 5 ml of bromothymol blue 0.04%) is added to the dish from a burette with continuous mixing until the gel is obtained (Shalan, 1974).

To remove any excess of acidity, the silica gel plates are dipped for 2 days in a current of tap water, then the plates are sterilized by flame.

D.6. CHLOROPHYLL (A) CONTENT

Chlorophyll (a) content is generally used as an index of growth of blue-green algae. Golterman and Cylmo (1968) developed the following procedure to estimate algal growth.

The algal growth is vigorously macerated together with washed sand. The chlorophyll (a) is then extracted with 90% v/v aqueous acetone. After centrifugation (500 rpm/min) the coloured supernatant is measured at a wave length of 665 nm using a one cm thick cuvette of the spectrophotometer (special zeiss, Jena) and 90% acetone as a blank. The amounts of chlorophyll (a) are then calculated as mg chlorophyll (a)/mg algal dry weight using the following equation:

$$\mu g \text{ chlorophyll (a)/mg dry wt} = \frac{Ex \times 11.9 \times \text{Volume of acetone}}{\text{Dry wt of the alga/mg}}$$

Where Ex = extension of 665 nm.

D.7. ALGAL BIOMASS IN SOIL

Lynn (1974) derived an equation to convert chlorophyll (a) to algal biomass:

Algal biomass = chlorophyll (a) mg/m^2 x 3.15 kg/ha

The method of determining algal biomass in soil involves:

i. The air dried algal crust of a limited area is homogenized in 10 ml of 90% acetone and extracted in the dark at 50°C for 24 hrs.

ii. The extract is cleared of debris by centrifuge spinning at full speed for 2-3 min.

iii. Solvent and contained chlorophyll are decanted to a spectrophotometer tube and read at 665 nm and 750 nm before and after acidifying with one drop of concentrated HCl.

iv. The corrected 665 nm readings are then used to calculate the concentration of chlorophyll (a) and algal biomass in the sample according to the following equation:

$$\text{Chlorophyll (a) } (mg/m^2) = \frac{26.73\,(665_b - 750_b) \times (665_a - 750_a)V}{A}$$

Algal biomass (kg/ha) = Chlorophyll (a) mg/m^2 x 3.15

V = volume in litres of extracting solution

A = area of the sample

665_b and 665_a are optional densities of 90% acetone extract before and after acidification, respectively.

D.8. CULTURE SOLUTIONS FOR BLUE-GREEN ALGAE

Various nutrient solutions have been used for isolation and maintenance of blue-green algae. Essentially all these solutions have the following characteristics:

i. Weakly alkaline reaction is usually obtained by the use of K_2HPO_4 or carbonate.

ii Nitrogen, if used, is in the form of Ca or KNO_3.

iii. Mg and Fe are present.

iv. Other elements are used.

v. The concentration of the different elements is variable with the different culture media.

Common media used with blue-green nitrogen-fixing algae are summarized as follows:

<u>Medium for tolypothrix (Watanabe, 1960) g/l</u>

KNO_3	3.0
$Na_2HPO_4.12H_2O$	0.5
$MgSO_4.7H_2O$	0.5
$CaCl_2$	0.02
$FeSO_4$	0.02
As solution	1 ml

Medium for *Anabaena variabilis* (Watanabe, 1960) g/l

KNO_3	2.02
K_2HPO_4	0.38
$MgSO_4.7H_2O$	0.25
KCl	0.30
NaCl	0.12

Mayers'C medium for blue-green algae (Watanabe, 1960)

KNO_3	1.0 g
K_2HPO_4	1.0 g
$MgSO_4.7H_2O$	0.25 g
$Ca(NO_3)_2.4H_2O$	0.025 g
Na citrate	0.165 g
$FeSO_4.7H_2O$	0.02 g
As solution	1 ml

El-Nawawy *et al* medium for blue-green algae (1958)

K_2HPO_4	0.3 g/l
$MgSO_4.7H_2O$	0.2 g/l
K_2SO_4	0.2 g/l
$CaCO_3$	0.1 g/l
glucose	2.0 g/l
$FeCl_3$ 1% (freshly prepared)	0.2 ml
Microelements solution (A_5)	1.0 ml
Distilled water	1000.0 ml
pH	7.5

A_5 micronutrient

H_3BO_3	2.9 g/l
$MnCl_2.4H_2O$	1.81 g/l
$ZnCl_2$	0.11 g/l
$CuSO_4.5H_2O$	0.08 g/l
Ammonium molybdate	0.018 g/l

Wieringa medium (1968)

K_2HPO_4	5.0 g/l
$MgSO_4.7H_2O$	5.0 g/l
$CaCl_2$	0.5 g/l
$NaCO_3$	0.5 g/l
Micronutrient solution	10 ml
Fe.EDTA (containing 50 ppm Fe)	10 ml
Distilled water	1000 ml

Microelement solution

H_3BO_3	3.10 g/l
$MnSO_4$	2.23 g/l
$ZnSO_4$	0.787 g/l
Na-molybdate	0.088 g/l
$CuSO_4$	0.125 g/l
Co-nitrate	0.146 g/l
KBr	0.119 g/l
KI	0.083 g/l
Distilled water	1000 ml

Tahas' medium (1963)

$MgSO_4$	0.25 g/l
K_2HPO_4	0.04 g/l
$CaCl_2.6H_2O$	0.0238 g/l
Na-citrate	0.165 g/l
$Fe_2(SO_4)_3$	0.02 g/l
Trace elements (A_5)	1.0 ml
Distilled water	1000 ml

Soil extract medium (The Botany School, Cambridge, 1966)

K_2HPO_4	0.02 g/l
$MgSO_4.7H_2O$	0.02 g/l
Soil extract	100.0 ml
Distilled water	900.0 ml

Soil extract is prepared by autoclaving 1 kg of garden soil with one litre of distilled water for ½ hour followed by filtration.

Bond's modified medium for blue-green algae (El-Borollosy, 1972)

KH_2PO_4	0.175 g/l
K_2HPO_4	0.075 g/l
$MgSO_4.7H_2O$	0.075 g/l
$NaNO_3$	0.250 g/l
$CaCl_2$	0.025 g/l
NaCl	0.025 g/l
$FeCl_3$(1%)	one drop
Microelements solution (A_5)	1 ml
Distilled water	1000 ml

Fogg's nitrogen free medium (Fogg, 1949)

KH_2PO_4	0.2 g/l
$MgSO_4$	0.2 g/l
$CaCl_2$	0.1 g/l
Na_2MO_4	0.1 mg
$MgCl_2$	0.1 mg
H_3BO_3	0.1 mg
$CaSO_4$	0.1 mg
$ZnSO_4$	0.1 mg
Fe-EDTA	1.0 ml
Distilled water	1000 ml

Gerloff's *et al* (1950) medium for blue-green algae

$NaNO_3$	0.0413 g/l
Na_2HPO_4	0.0082 g/l
KCL	0.0086 g/l
$MgCl_2.6H_2O$	0.0209 g/l
Na_2SO_4	0.0146 g/l
$CaCl_2.2H_2O$	0.0359 g/l
Fe-citrate	0.003 g/l
Citric acid	0.003 g/l
$NaCO_3$	0.02 g/l
Na_2SiO_3	0.025 g/l

Hogland's solution H-Z is added to the medium 1/25 of strength specified for higher plants.

Chu's medium 10 (1942)

$Ca(NO_3)_2$	0.04 g/l
K_2HPO_4	0.01-0.005 g/l
$MgSO_4.7H_2O$	0.025 g/l
Na_2CaO_3	0.02 g/l
$NaSiO_3$	0.025 g/l
$FeCl_3$	0.008 g/l

Allen and Arnon's medium (1955) for blue-green algae

$MgSO_4$	0.001 M
$CaCl_2$	0.0005 M
NaCl	0.004 M
K_2HPO_4	0.002 M

Micronutrients

Fe (as EDTA complex)	4.0 ppm
Mn (as $MnSO_4.4H_2O$)	0.5 ppm
Mo (as MoO_3)	0.1 ppm
Zn (as $ZnSO_4.4H_2O$)	0.05 ppm
Cu (as $CuSO_4.5H_2O$)	0.02 ppm
B (as H_3BO_4)	0.50 ppm
V (as NH_4VO_3)	0.01 ppm
Co (as $Co(NO_3)_2.6H_2O$)	0.01 ppm
Ni (as $NiSO_4.6H_2O$)	0.01 ppm
G (as $G_2(SO_4)_2K_2SO_4.24H_2O$)	0.01 ppm
W (as $Na_2WO_4.2H_2O$)	0.01 ppm
Titanium oxide	0.02 ppm

0.02 M KNO_3 may be added where a nitrogen source is required.

Modified Z medium (Staub, 1961)

$NaNO_3$	46.7 mg
$Ca(NO_3)_2.4H_2O$	59.0 mg
K_2HPO_4	62.0 mg
$MgSO_4.7H_2O$	25.0 mg
Na_2CO_3	21.0 mg
Fe EDTA solution	10.0 ml
Trace element solution	0.08 ml
Distilled water up to	1000 ml

EDTA solution

5 ml of 0.1 N solution of $FeCl_3.6H_2O$ in 0.1 N HCl solution of disodium salt of ethylenediamine tetra acetic acid are mixed together and then topped up to 500 ml.

Trace elements solution: 100 ml contain, mg

H_3BO_3	310.0
$MnSO_4.4H_2O$	223.0
$Na_2SO_4.2H_2O$	3.3
$(NH_4)_6MO_7.4H_2O$	8.8
KBr	11.9
KI	8.3
$ZnSO_4.7H_2O$	28.7
$Cd(NO_3)_2.4H_2O$	15.4
$Co(NO_3)_2.6H_2O$	14.6
$CuSO_4.2H_2O$	12.5
$NiSO_4(NH_4)_2SO_4.6H_2O$	19.8
$C_2(NO_3)_2.7H_2O$	3.7
$V_2O_4(SO_4)_3.16\ H_2O$	3.5
$Al_2(SO_4)_3K_2SO_4.24H_2O$	47.4

D.9. MANAGEMENT OF ALGAL PRODUCTION FOR PADDY INOCULATION
(Indian Technology)

Singh (1978) outlined the following recommendation for the production and application of blue-green algae:

Algal Propagation

i. Prepare shallow trays of galvanized iron sheet (4' x 3' x 9") or of bricks and mortar so as to have permanent units. The size and number of trays (tanks) depends upon the amount of inoculum produced. It is advisable to have permanent structures with proper irrigating and draining facilities at the block level.

ii. Keep 17 kg of soil in the trays and mix it well with 10 g of superphosphate. About 2 g of Na molybdate may alsobe added if there is a response to its addition in a particular type of soil.

iii. Keep the soil flooded after the addition of water. Lime is recommended for acidic soils to raise the pH.

iv. When the water becomes clear sprinkle the starter culture on the surface of the standing water. The starter culture must be in a healthy condition (about 20 g fresh material equivalent to 2 g dry wt.), for rapid growth. The trays should be kept in the open field since algae grown under field conditions multiply rapidly after inoculation.

v. Algae multiply rapidly during the summer and cover the area in 15 days. Harvest the algal mat and use it for inoculation. In off seasons the algal mat is collected, dried in the shade and is kept for further possible use. As far as possible fresh algae should be used as inoculum.

vi. Continue cultivation and harvesting with the above amendments and change the soil after 3-4 harvests. A single harvest from a tray 85 cm x 65 cm x 15 cm, after 20 days, weighs around 250-400 g of fresh algae.

vii. To control the insects which eat blue-green algae add 3-5 g of diazinon or cyeolane or furadan.

viii. Nitrogen-fixing blue-green algae growing in fallow flooded rice fields before ploughing can also be collected and used as a starter culture or inoculum. For this purpose some experience of identifying promising blue-green algae by visual observation is required.

Field Application

i. Inoculate fresh algal material as fast as possible, rather than dried algae, at the rate of 25-50 kg/ha (90% moisture) on standing water which corresponds to 3-5 kg/ha on a dry weight basis. The addition of excess algal material helps to produce more algae in a shorter time.

ii. Apply algal material with each crop for 3-4 consecutive seasons.

iii. Inoculate the algae after a week of planting in clear water. Inoculation should be avoided during rains.

iv. Apply superphosphate at the rate of 20-40 kg P_2O_5/ha in two doses during inoculation. The second one should be applied after 20 days.

v. Algae can be used with 20-40 kg N-fertilizer safely to obtain higher yields.

vi. Recommended rates of pesticides or herbicides and other agronomic practices do not generally interfere with algal establishment.

AZOLLA

E.l. GUIDELINES FOR AZOLLA MULTIPLICATION AND UTILIZATION

Singh (1978, personal communication) suggested a few guidelines for the management of Azolla. (Fig. 29).

Multiplication

i. Ploughed, levelled and bunded fields must be used for multiplication.

ii. Maintain 5-10 cm standing water continuously while multiplication is in progress. Raising the water level to 30 cm will not have any adverse effect.

iii. Inoculate Azolla at the rate of 0.2 to 0.4 kg per m^2 depending upon the availability of inoculum.

iv. Mix superphosphate at the rate of 4 to 20 kg P_2O_5/ha together with inoculum. Ratna phosphate and rock phosphate are not recommended for Azolla cultivation. A few grams (0.1 to 1.0 g/kg of pesticide furadan) should also be mixed with the inoculum or both superphosphate and furadan may be applied after inoculation.

Fig. 29 *Azolla* multiplication
(contributed by P.K. Singh)

v. After the formation of a layer (in a week) harvest the Azolla with the help of a bamboo stick and again inoculate at the same rate for further multiplication.

vi. Harvested Azolla should be re-inoculated in another field otherwise it will decompose and turn into compost (5% N) which can also be used.

vii. Generally fresh Azolla (0.2 - 0.3% N) is used for manuring.

viii. Under conditions at Cuttack (India) Azolla multiplies throughout the year while the water temperature is between 17 - 29 and 28 - 33°C (night/day) at the rate of 2 - 4 times per week. Its multiplication is affected adversely when the water temperature exceeds 32-40°C (night/day).

ix. Azolla grows well in soils of slightly acid to alkaline pH (6 - 8). Very acid soils of Kerala (pH 2.9 - 3.6) do not support its growth.

x. It can also be multiplied in concrete tanks containing 15 cm of soil.

xi. The multiplication programme should be started about a month in advance so as to have sufficient inoculum for one acre.
The multiplication programme may be summarized as follows.
4 kg azolla + 36 g superphosphate (SP) inoculate 4 x 3 m incubate one week 12 kg azolla + 90 g SP inoculate 5 x 6 m incubate one week 36 kg azolla + 300 g SP inoculate 10 x 10 m incubate one week 110 kg azolla + 900 SP inoculate 20 x 15 m incubate one week 330 kg azolla + 3 kg SP inoculate 45 x 20 m incubate one week 1000 kg azolla inoculate into one acre with SP.
(Note: A few grams of the pesticide furadan may be mixed with each inoculum).

E.2. MULTIPLICATION OF AZOLLA ON LARGE AREAS

i. Divide fields into (not larger than) 300 or 400 m^2 subplots. Azolla grows better in smaller plots since it gets blown about by wind if the plots are too large.

ii. When pests occur apply 2 - 3 kg/ha of furadan on the spots where damage has started. Azolla pests do not attack rice plants and in fact help to foster Azolla decomposition.

E.3. UTILIZATION OF AZOLLA

<u>As Green Manure</u>:

Grow Azolla before planting crops in ploughed and levelled fields, for which inoculant required is about 2000 kg/ha. After 7-14 days, drain the field and mix the Azolla layer in the soil, with a plough if possible. Then transplant rice seedlings into the plot within a week. Application of a second layer of Azolla after incorporation of the first will provide more nitrogen. One layer of Azolla provides 10 tonnes green matter/ha, which is equivalent to 25-30 kg N. The increase in yield ranges from 0.5 to 1.5 tonnes/ha over control.

As Dual Cropping with Rice

If water is not available before planting, inoculate the Azolla on standing water at the rate of 500 to 1 000 kg/ha a week after planting when seedlings are established. Recommended doses of superphosphate for rice may be applied in split doses, i.e. the first half as a basic dose and the second half before addition of Azolla with furadan. After the mat has formed, drain the field, if possible, and incorporate the Azolla with the paddy water. If this is not possible, leave it and it will decompose in due course and increase the crop yield. It is advisable to use a basal dose of nitrogen fertilizer when the Azolla is grown with rice to encourage better tillering. Floating Azolla also checks weed growth.

Use of Azolla with Inorganic Nitrogen Fertilizer

Azolla is also used along with nitrogen fertilizer. Both of them are used as a basic dressing or as topdressing to obtain maximum yield.

E.4 NUTRIENT SOLUTIONS USED FOR AZOLLA GROWTH

Espinas and Watanabe (1976)

P	20 ppm
K	40 ppm
Ca	40 ppm
Mg	40 ppm
Mn	0.5 ppm
Mo	0.15 ppm
B	0.2 ppm
Zn	0.01 ppm
Ca	0.01 ppm
Fe (as ferric citrate)	2 ppm
pH	5.5

Le Van Can, 1963 (Tuan and Thuyet, 1978)

1/10	Hellrigel solution
1/10 to 1/5	Knop solution

Tuan and Thuyet (1978)

1/10	Knop solution
paddy water + 120 mg/1	K_2HPO_4

Vo Minh Kha, 1971 (Tuan and Thuyet, 1978)

KH_2PO_4	6.8 mg/1
KCl	7.5 mg/1
$MgSO_4$	6.0 mg/1
$CaCl_2$	33.6 mg/1
K_2SO_4	4.3 mg/1
$FeCl_3H_2O$	2.5 mg/1
Microelements	1.0 mg/1
pH	5.5-6.0

Chu (1978) Chekiang Agr, 6302

i. N-free solution

$CaSO_4.2H_2O$	0.1717 g
$MgSO_4.7H_2O$	0.0416 g
$FeCl_3$	0.0021 g
H_3BO_3	0.0003 g
NaH_2PO_4	0.0416 g
KCl	0.0228 g
H_2MoO_4	0.0003 g
Distilled water	1 000 ml

ii. N-Culture solution

Add 1 000 ppm of NH_4NO_3 to Chekiang Agr. 6302.

Appendix F

CULTURE COLLECTIONS OF NITROGEN-FIXING ORGANISMS

The following list of addresses covers most centres carrying nitrogen-fixing organisms

F.l. CULTURE COLLECTIONS OF RHIZOBIUM

Australia

Mr. J. Brockwell
CSIRO, Division of Plant Industry
Box 1600 Canberra City, A.C.T.

Dr. R.J. Roughley
AIRC Service
Dept. of Agriculture, PMB 10
Rydalmere, N.S.W. 2116

Dr. C.A. Parker
Dept. of Soil and Plant Nutrition
Institute of Agriculture
Nedlands, W. Australia, 6009

Brazil

Dr. J. Dobereiner
Institute de Pesqurae Experimentacae
do Sentro sul (IPEACS)
Km 47 via Campo Grande Zc-26, GB.

Canada

Dr. M. Hauser
Dept. of Microbiology
University of Guelf

Dr. F.B. Roslycky
Res. Institute, Canada Agriculture
University Sub. P.O.
London, Ontario

Czechoslovakia

Dr. Helena Jakubcova
Dept. of Microbiology
Central Res. Inst. of Plant Production
16106 Praha 6, Ruzynev, 507

France

Dr. M. Obaton
Station de Recherches de Microbiologie des Sols,
7 rue Sully - 21 - Dijon

India

Dr. R.B. Patil
Dept. of Microbiology
University of Agricultural Sciences
Bangalore 24

Dr. R.B. Rewari
IARI
Division of Microbiology
New Delhi, 12

Dr. M. Sulaiman
BNF Scheme
Poona - 5

Japan

Dr. S. Ishizawa
Dept. of Soils and Fertilizers
National Inst. of Agricultural Sciences
Nishigahara, Kita-kir, Tokyo

Dr. S. Yoshida
Dept. of Agricultural chemistry
Nagoya University
Chekusa-Ku, Nagoya

The Netherlands

Miss E.O. Biewenga
Institute of Soil Fertility
Oosterweg 41, Haren, Groningen

Dr. E.G. Mulder
Laboratory of Microbiology
University of Wageningen, Wageningen

Poland

Dr. Z. Lorkiewicz
Institute of Microbiology and Biochemistry
University of Marie Curie
Skiodowska, Lublin

Rhodesia

Dr. W.P.L. Sandman
Glasslands Res. Station
P. Bag 701, Marandelles
Rhodesia, Africa

South Africa

Dr. B. Strijdom
Plant Protection Res. Institute
Private Bag 134
Pretoria, S-Africa

United Kingdom

Dr. D. Gareth Jones
Dept. of Agricultural Botany
University College of Wales
Panglais, Aberystwyth, Wales

Dr. M. Dye
Rothamsted Experimental Station
Harpenden, Hertfordshire

U.S.A.

Dr. B.E. Caldwell
USDA
Crops Res. Division
Beltsville, Maryland 20705

Dr. F.E. Lessel
ATCC
12301 Parklawn Dr.
Rockville, Md, 20852

U.S.S.R.

Dr. F.S. Matevosian
Institute of Microbiology
Armenian S.S.R. Academy of Sciences
Tsharentso 19,
Erevan 25, Armenian S.S.R.

Dr. L.M. Dorossinsky
Al1 Union Scientific Res. Institute of Agric. Microbiology
Leningrad, Gerzen St. 42

Yogoslavia

Dr. Vera Petrovic
Institute of Soil Science
Beograd, Teodova Drajzera 7

In addition to the above collection centres, there are two MIRCENS which are dealing with rhizobia cultures:

i. Porto Alegre (Brazil)
Dr. J.R. Jardin Freire
UFRGS Faculdade de
Agronomia,Caixa Postal 776
90000 Porto Alegre, Brazil.

ii. Dr. S.O. Keya
Soil Science
University of Nairobi
P.O. Box 30197
Nairobi, Kenya.

F.2. CULTURE COLLECTIONS OF BLUE-GREEN ALGAE

Canada

National Res. Council
Ottawa, Canada

Czeckoslovakia

Prof. B. Fott
Charles University
Chair of Botany, Cryptogamological Dept.
Praha

Dr. J. Komarek, Dr. O. Lhotsky
Czechoslovak Academy of Sciences
Botanical Institute
Laboratory of Hydrobotanics
Treban

Prof. S. Prat
Czechoslovak Academy of Sciences
Institute of Experimental Botany
Collection of Cultures of Autotiophic Organisms
Praha

Federal Republic Germany

Dr. W. Koch
Universitat Gottingen
Planzenphysiologisches Institut
Gottingen

France

Dr. R. Pourriot
Centre de Recherches Hydrobiologiques
Gif - sur - Yvette

Prof. R.Y. Stanier
Institut Pasteur
25 Rue du Doteur Roux
XVe Arrond, Paris

German Democratic Republic

Prof. H. Borriss
Ernst-Moritz-Arndt Universitat
Botanisches Institut
Greifswald

United Kingdom

Dr. E.A. George
The Culture Centre for Algae and Protozoa
36 Storeys Way, Cambridge

India

Prof. G.S. Venkataraman
Indian Agricultural Res. Institute
Culture Collection of Microalgae
New Delhi

Prof. T.V. Desikachary
University of Madras
Centre for Advanced Study in Botany,
Madras

Japan

Dr. I. Ichimura
University of Tokyo
Institute of Applied Microbiology
The Algal Collection
Tokyo

Philippines

Dr. J. Watanabe
IRRI
Los Banos, Philippines

U.S.A.

Prof. R.C. Starr
Indiana University
Culture Collection of Algae
Bloomington, Indiana

Prof. R.W. Castenholz
University of Oregon
Department of Biology
Eugene, Oregon

U.S.S.R.

Dr. B.V. Gromov
State University of Leningrad
Institute of Biology
Leningrad - Petergol
U.S.S.R.

F. 3. CULTURE COLLECTIONS OF AZOTOBACTER AND AZOSPIRILLUM

Holland

Dr. J.H. Becking
Institute for Atomic Sciences in Agriculture
Potsbus 48. Wageningen

Brazil

Dr. F. Dobereiner
Institute de Pesqurae Experimentacae
do Sento Sul (IPEACS)
Km 47 Via Campo Grande Zc-26 GB

United Kingdom

Dr. J. Postgate
Unit of Nitrogen Fixation
ARC University of Sussex
Brighton

F. 4. CULTURE COLLECTIONS OF AZOLLA

U.S.A.

Dr. G.A. Peters
C.F. Kettering Lab
Yellow Springs, Ohio 45387

Holland

Dr. J.H. Becking
Institute for Atomic Sciences in Agriculture
Posbus 48, Wageningen

India

Dr. P.K. Singh
Central Rice Res. Institute
Cuttack, Orissa

Philippines

Dr. I. Watanabe
The International Rice Res. Institute
Los Banos, Laguna

China

Dr. L.C. Chu
Soil & Fertilizer Res. Institute
Fukien Acadamy of Agriculture Sciences

REFERENCES

Abd-el-Gaffar, S.A.M. Some factors affecting nodulation and nitrogen-fixation of soybean (Glycine max). M Sc Thesis. Faculty of Agriculture, University of Alexandria. 1976

Abdel-Malek, Y. and Ishac, Y.Z. Evaluation of methods used in counting Azotobacters. J. Appl. Bacteriol., 31:267-275. 1968

Abdel-Samea, M.E. Seed pelleting as a procedure for legume seed inoculation. M Sc Thesis, University of Cairo, Faculty of Agriculture, Giza, Egypt. 1975

Abdel-Wahab, S.M. Genetic control of nitrogen-fixation in Rhizobium trifolii strains. Ph D Thesis, University of Cairo, Faculty of Agriculture, Cairo, Egypt. 1977

Abou-el-Fadl, M., El-Nawawy, M.S., El-Mofty, M., El-Nadi, M., and Farag, F. N-fixation by the blue-green alga, Tolypothrix tenuis as influenced by ammonium sulphate, compost, straw and superphosphate with special reference to its effect on rice yield. J. Soil Sci., U.A.R. 49:91-104. 1964

Abou-el-Fadl, M., Eid, M.T., Hamissa, E.M., El-Nawawy, A.S., and Shoukry, A. The effect of the N_2-fixing blue-green alga Tolypothrix tenuis on the yield of paddy. J. Microbiol, U.A.R. 2:241-249. 1967

Abou-el-Fadl, M., Hamissa, M.R., El-Nawawy, A.S., and Abdel-el-Aziz, M.S. Evaluation of the blue-green alga Tolypothrix tenuis as nitrogen source for rice plants. The agric. Conf. of Rice, Cairo (In Arabic). l972

Aiyer, R.S., Salahudeen S., and Venkataraman, G.S. Long term algalization field trials with high yielding rice varieties. Indian J. Agric. Sci. 42:380-383. 1972

Akkermans, A.D.L. Nitrogen-fixation and nodulation of Alnus and Hippophae under natural conditions. Ph D Thesis, University of Leiden. 1971

Akkermans, A.D.L. Root nodule symbioses in leguminous N_2-fixing plants. In: interactions between non-pathogenic soil microorganisms and plants (eds. Y.R. Dommergues and S.V. Krupa) 335-372, Elsevier Scientific Pub. Co., Amsterdam. 1978

Akkermans, A.D.L., Abdul Kadir, S., and Trinick, M.J. Nz-fixing root nodules in Ulmaceae: Parasponia or (and) Trema spp. Plant and Soil. 1978

Ala el-Din, M.N. Nitrogenase activity of soybean nodules as affected by different levels of soil salinity. Ist. Conf. Microbiol. Soc. Iraq, Baghdad 5-7 Oct. 1976

Alexander, M. Ecology of nitrogen-fixing organisms. In:A. Ayanaba and P.J. Dart (eds.) Biological nitrogen-fixation in farming systems in the tropics. John Wiley and Sons. 99-114. 1975

Alexander, M. Soil microbiology 2nd edition, J. Wiley and Sons. New York, London. 1977

Alexander, V.A. Nitrogen-fixation by blue-green algae in polar and subpolar regions. In: Nitrogen-fixation by free-living microorganisms. (ed. W.D.P. Stewart). Cambridge University Press. 175-188. 1976

Allen, M.B. 1956 Photosynthetic nitrogen-fixation by blue-green algae. Science Monthly. 83:100-106.

Allen, M.B., and Arnon, D.I. 1955 Studies on nitrogen-fixing blue-green algae. Growth and nitrogen-fixation by Anabaena cylindrica Lemon Pl. Physiol. 30:366-372.

Allen, O.N. 1959 Experiments in soil bacteriology. Burgess Pub. Co., Minn. Minnesota.

Anellis, A., Berkowitz, D., and Kemper, D. 1973 Comparative resistance of non-sporogenic bacteria to low temperature, gamma irradiation. Appl. Microbiol. 25:517.

Ashton, P.J. 1974 The effects of some environmental factors on the growth of Azolla filiculoides Lam. In: The orange river progress report. (Inst. for Environmental Sciences), University of the OFS Bloemfontein, South Africa. 123-138.

Ashton, P.J., and Walmsley, R.D. 1976 The aquatic fern Azolla and its Anabaena symbiont. Endeavour. 35:39-43.

Aziz, R.Kh. 1978 A study on the industrial production of the Genus Rhizobium and the effect of some chemical pesticides on its growth activity. M Sc Thesis, University of Baghdad, Iraq.

Barker, K.R., Huisingh, D., and Johnston, S.A. 1972 Antagenistic interaction between Heterodera glycines and Rhizobium japonicum on soybean. Phytopathol. 62 (10):1201-1205.

Becking, J.H. 1961 Studies on the nitrogen-fixing bacteria of the genus Beijerinckia. Geographical and ecological distribution in soil. Plant and soil. 14:49-81.

Becking, J.H. 1963 Fixation of molecular nitrogen by an aerobic Vibrio or Spirillum. Ant. Van Leeuwenhoek, 29:326.

Becking, J.H. 1968 Nitrogen-fixation by non-leguminous plants. Proc. Symp. Nitrogen in Soil. Groningen 1967, Nitrogen. Dutch Nitrogenous Fertilizer Rev. 12:47-74.

Becking, J.H. 1972 Va. Symbiosen: Stickstoff-bindung. Fortschritte der Botanik. 34:459-467.

Becking, J.H. 1974 a Azotobacteriaceae. In: Bergey’s Manual of Determinative Bacteriology, 8th edition (eds. R.E. Buchanan and N.E. Gibbons). The Williams and Wilkins Co., Baltimore. 253-261.

Becking, J.H. 1974 b Family III, Frankiaceae Becking 1970. In: Bergey’s Manual of Determinative Bacteriology, (eds. R.E. Buchanan and N.E. Gibbons). The Williams and Wilkins Co., Baltimore, Md. 701-706.

Becking, J.H. 1976 Contribution of plant algae associations. In: Proc. of the 1st International Symposium on Nitrogen-Fixation. (eds. W.E. Newton and C.J. Nyman). Washington State University Press, Pullman. Vo1.2:556-580.

Becking, J.H. 1977 Dinitrogen fixing associations in higher plants other than legumes. In: A treatise on dinitrogen-fixation section III, biology (eds. R.W.F. Hardy and W.S. Silver). Wiley-Interscience.Pub. London, Sydney, Toronto. 185-275.

Becking, J.H. 1978 a Ecology and physiological adaptations of Anabaena in the Azolla-Anabaena azolla symbiosis. Ecol. Bull. (Stockholm) 26:266-281.

Becking, J.H. 1978 b Beijerinckia in irrigated rice soils. Ecol. Bull. (Stockholm) 26:116-129.

Becking, J.H. Environmental requirements of Azolla for use in tropical rice production.
1979 c Symposium on Nitrogen and Rice. 18-21 September 1978. IRRI Los Banos, Laguna, Philippines.

Bergersen, F.J. Physiology of legume symbiosis. In: Interactions between non-pathogenic
1978 soil microorganisms and plants. (eds. W.R. Dommergues and S.V. Krupa). Elsevier Sci. Pub. Co., Amsterdam, Oxford. 305-333.

Bergersen, F.J., Kennedy, G.S., and Wittmann, W. Nitrogen-fixation in the coralloid roots
1965 of Macrozamia communis L. Jhonson. Aust. J. Biol. Sci. 18:1135-42.

Blackburn, R.D., and Welden, L.W. The sensitivity of duckweed (Lamnaceae) and Azolla to
1965 diquat and paraquat. 13:147-149.

Bond, G. The incidence and importance of biological fixation of nitrogen. Adv. Sci.
1959 15:382-386.

Bond, G. The root nodules of non-leguminous angiosperms. In: Symbiotic associations, l3th
1963 symp. Soc. Gen. Microbiol., (eds. P.S. Nutman and B. Mosse). Cambridge Univ. Press. London. 72-91
.
Bond, G. Fixation of nitrogen by higher plants other than legumes. Ann. Rev. Pl. Physiol.
1967 18:107-126.

Bond, G. Root nodule symbioses with actinomycete-like organisms. In: The biology of
1974 nitrogen-fixation in plants (ed. A. Quispel). North-Holland Publishing Co., Amsterdam. 342-378.

Bond, G. The results of the IBP survey of root-nodule formation in non-leguminous angio-
1976 sperms. In: Symbiotic nitrogen-fixation in plants. (ed. P.S. Nutman). Cambridge Univ. Press. Cambridge. 443-474.

Bone, D.H. Relationship between phosphates and alkaline phosphatase of Anabaena flos-aquae
1971 a in continuous culture. Arch. Mikrobiol. 80:147-153.

Bone, D.H. Kinetics of synthesis of nitrogenase in batch and continuous culture of
1971 b Anabaena flos-aquae. Arch. Microbiol. 80:242-251.

Bortels, H. Uber die Bedeutung des Molybdans fur stickstoffbindende Nostoccaceen
1940 Arch. Mikrobial. 11:155-186.

Brock, R.D. Halophilic blue-green algae. Arch-Microbiol. 107-109.
1976

Brockwell, J. Application of legume seed inoculants. In: A treatise on dinitrogen fixation, section IV agronomy and ecology. (eds. R.W.F. Hardy and A.H. Gibson). A. Wiley- Interscience Pub. New York, London. 277-310.

Brockwell, J., and Gault, R.R. Evaluation of Symbiotic response in 21 introduced lines of
1972 chick pea (Cicer arietinum L.) Pl. Introduction. Rev. Div. Pl. Ind., CSIRO Aust. 9 (1):4-11.

Brotonegoro, S., and Abdulkadir, S. Growth and nitrogen-fixing activity of Azolla pinnata.
1976 Annales Bogorienses. 6(2):69-123.

Brown, M.E., Burlingham, S.K., and Jackson, R.M. Studies on Azotobacter species in soil, II.
1962 Population of Azotobacter in the rhizosphere and effects of artificial inoculation. Plant and Soil.l7:309-319.

Brown, M.E., Burlingham, S.K., and Jackson, R.M. Studies on Azotobacter species in soil,
1964 III. Effects of artificial inoculation on crop yields. Plant and Soil.20:
194-214.

Brown, M., Jackson, R.M., and Burlingham, S.K. Effects produced on tomato plants,
1968 Lycopersicum esculentum, by seed or root treatment with gibberillic acid.
J. Exptl Bot. 19:544-552.

Buckingham, K., Ela, S., Morris, J., and Goldman, C. Protein quality evaluation and
1977 proximate analysis of Azolla filiculoides. Fed. Proc. Am. Soc. Exp. Biol.
36:4802.

Burns, R.C., and Hardy, R.W.F. Nitrogen-fixation in bacteria and higher plants. Springer-
1975 Verlag Berlin. Heidelberg. New York.

Burris, R.H. A synthesis paper on non-leguminous N_2-fixing system In: Recent developments
1977 in nitrogen-fixation (eds. W.E. Newton, J.R. Postgate and C. Rodriguez-
Barruaco). Academic Press, London. New York. San Fransisco. 487-513.

Burris, R.H., Okon, Y., and Albrecht, S.L. Properties and reactions of Spirillum lipoferum,
1978 Ecol. Bull. (Stockholm) 26:353-363.

Burton, J.E. Rhizobium culture and use. In: Microbiol technology (ed. H.J. Peppler).
1967 Van Nostrand Reinhold, New York. 1-33.

Burton, J.C. Nodulation and symbiotic nitrogen-fixation. In: Alfalfalfa science and
1972 technology, Monograph 15 (ed. C.H. Hanson). Amer. Soc. Agron, 677
S. Segoe Road, Modison, Wisconsin.

Burton, J.C. Problems in obtaining adequate inoculation of soybeans. In: World soybean
1976 research (ed. L.D. Hill). Proc. of the World Soybean Res. Conference.

Caldwell, B.E. Initial competition of root nodule bacteria on soybeans in a field
1969 environment. Agron. J. 61:813-815.

Cast Effect of increased nitrogen-fixation on stratospheric zone. Council for
1976 Agric. Sci. Techn. Report No.53.

Chhonkar, P.K., and Negi, P.S. Response of soybean to rhizobial inoculation with different
1971 strains of Rhizobium japonicum. Indian J. Agric. Sci. 41:741-744.

Chowdhury, M.S. Response of soybean to Rhizobium inoculation at Morogorro, Tanzania. In:
1975 Biological nitrogen-fixation in farming systems of the tropics. (eds.
A. Ayanaba and P.J. Dart). John Wiley and Sons. 245-253.

Chowdhury, M.S., Marshal, K.C. and Parker, C.A. Growth rates of R. trifolii and R. lupini
1968 in sterilized soil. Aust. J. Agric. Res. 19:919-925.

Chu, L.C. The use of azolla in rice production in China. Symposium on Nitrogen and Rice,
1979 18-21, September 1978. IRRI, Los Banos, Laguna, Philippines.

Chu, S.P. The influence of mineral composition of the medium on the growth of planktonic
1942 algae, 1. Methods and culture media. J. Ecol. 30:284-325.

Cohn, J., and Renlund, R.N. Notes on Azolla caroliniana. Am. Fern. J. 43:7-11.
1953

Coler, R.A., Gunner, H.B., and Zatyrka, S.A. Bacteria-free algae. Bact. Proc. 28.
1969

Dadhich, K.S., Varma, A.K., and Venkataraman, G.S. Effect of Calothrix inoculation on
1969 vegetable crops. Plant and Soil. 31:377.

Daff, M.J., Begg, J., and Stewart, W.D.P. A virus of blue-green algae from fresh water
1970 habitats in Scotland. New Phytol. 69:1029-1038.

Damirgi, S.M., and Johnson, H.W. Effect of soil actinomycetes on strains of R. japonicum.
1966 Agron. J. 58:223-224.

Dart, P. Infection and development of leguminous nodules. In: A treatise on dinitrogen
1977 fixation: Section III Biology. (eds. R.W.F. Hardy and W.S. Silver).
A. Wiley Interscience Pub. London, Sydney, Toronto. 367-472.

Dart, P.J. Groundnut microbiology program at ICRISAT. Presentation to the Quinquennial
1978 Review, 6 October l978.

Dart, P.J., and Mercer, F.V. The effect of growth temperature, level of ammonium nitrate
1965 and light intensity on the growth and nodulation of cowpea (Vigna sinensis
Endl. Ex. Hassk.) Aust. J. Agric. Res. 16 (3):32l-45.

Date, R.A. Principles of strain selection. In:Symbiotic nitrogen fixation in plants.
1976 (ed. P.S. Nutman). Cambridge Univ. Press, Cambridge, England. 137-150.

Date, R.S., and Roughley, R.J. Preparation of legume seed inoculants. In: Treatise on
1977 dinitrogen fixation. Section IV. Agronomy and Ecology. (eds. R.W.F. Hardy
and A.H. Gibson). 243-276.

Day, J.M. Nitrogen-fixing associations between bacteria and tropical grass roots. In:
1975 Biological nitrogen-fixation in farming systems of the tropics. (ed.
S.A. Ayanaba and P.J. Dart). J. Wiley and Sons, New York, Brisbane, Toronto.
273-288.

Day, J.M., and Dobereiner, J. Physiological aspects of N_2-fixation by Spirillum and
1975 Digitaria roots. Soil. Biol. Biochem. 7:45-50.

De, P.K. The role of blue-green algae in nitrogen-fixation in rice fields. Proc. Roy.
1939 Soc. London. 127B: 121-39.

De, P.K., and Mandal, L.N. Fixation of nitrogen by algae in rice soils. Soil Sci.
1956 81:453-458.

De, P.K., and Sulaiman, M. Fixation of nitrogen in rice soils by algae as influenced by
1950 crop, carbon dioxide and inorganic substances. Soil Sci. 70:137.

Department of Agriculture, Canton Province, China. Questions and answers concerning Azolla
1975 cultivation. Peoples Publisher, Canton, 41.

Derx, H.G. Beijerinckia, a new genus of nitrogen-fixing bacteria occurring in tropical
1950 soils. Konink. Ned. Akad. Wetenschnap. Proc. 53:140-147.

Desikachary, T.V. Status of classical taxonomy. In: The biology of blue-green algae.
1973 (eds. N.G. Car and B.A. Whitton). Blackwell Scientific Pub. Oxford, London.
473-481.

Diatloff, A. Larvae of Rivellia Sp. (Diptera, Platystomatidae) attacking the root nodules
1965 of Glycine javanica L. J. Ent. Soc. Qd. 4:86.

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

Dobereiner, J. Nitrogen-fixing bacteria of the genus Beijerinckia Derxia in the
1961 rhizosphere of sugar cane. Plant and Soil. 14:211-217.

Dobereiner, J. Azotobacter paspali n. Sp. Uma bacteria fixadora de nitrogenio na rhizopera
1966 de paspalum. Pesq. Agrop. Bras. I:357-365.

Dobereiner, J. Nitrogen-fixation in the rhizosphere of tropical forage grasses. Global
1975 Impacts of Applied Microbiology IV Sao Paulo, Brazil.

Dobereiner, J. N_2-fixation association with non-leguminous plants. In: Genetic engineering
1977 for nitrogen-fixation. (ed. A. Hollaender). Plenum Press, New York and
London. 451-461.

Dobereiner, J. Influence of environmental factors on the occurrence of Spirillum lipoferum
1978 in soils and roots. Ecol. Bull. (Stockholm) 26:343-352.

Dobereiner, J., and Campelo, A.B. Non-symbiotic nitrogen-fixing bacteria in tropical soils.
1971 In: Biological nitrogen-fixation in natural and agricultural habitats. Plant
and Soil. Special Volume. (eds. T.A. Lie and E.G. Mulder). The Hague,
Myhoff. 457-470.

Dobereiner, J., and Campelo, A.B. Importance of legumes and their contribution to tropical
1976 agriculture. In: A treatise on dinitrogen fixation. Section IV Agronomy and
Ecology. (eds. R.W.F. Hardy and A. Gibson). Wiley Interscience Pub.
New York, London. 191-220.

Dobereiner, J., Day, J.M., and Dart, P.J. Nitrogenase activity and oxygen sensitivity of
1972 a the Paspalum notatum Azotobacter paspali association. J. Gen. Microbiol.
71:103-116.

Dobereiner, J., Day, J.M., and Dart, P.J. Nitrogenase activity in the rhizosphere of
1972 b sugar cane and other tropical grasses. Plant and Soil 37:191-196.

Dommergues, Y. Evaluation de loux de fixation de l’azote dans un Sol dunaire reboise en
1963 filas Casuarina equesetifolia Agrochimica.7:335-340.

Dommergues, Y.R. Impact on soil management and plant growth. In: Interactions between
1978 non-pathogenic soil microorganisms and plants. (eds. Y.R. Dommergues and
S.V. Krupa). Elsevier Sci. Pub. Co., Amsterdam, Oxford. 443-458.

El-Borollosy, M.A. Studies on N-fixing blue-green algae in A.R.E. M Sc Thesis, Fac. Agric.,
1972 Ain Shams Univ., Cairo, ARE.

El-Nawawy, A.S., Ibrahim, A.N., and Abou-el-Fadl, M. Nitrogen-fixation by Calothrix sp
1968 as influenced by certain sodium salts and nitrogenous compounds. Acta argon.
hung. 17:323-327.

El-Nawawy, A.S., Kamal, R.M., and Abou-el-Fadl, M. Growth and N-fixation by blue-green
1972 alga, T. tenuis as affected by phosphorus content of media. Agric. Res.
Rev. 50:111-115.

El-Nawawy, A.S., Lotfi, M., and Fahmy, M. Studies on the ability of some blue-green algae
1958 to fix atmospheric nitrogen and their effect on growth and yield of paddy.
Agric. Res. Rev., UAR, 36:308-349.

El-Nawawy, A.S., Hamdi, Y., El-Sayed, M., and Shaalan, S.N. Effect of cobalt and
1970 molybdenum on growth and N-fixation by T. tenuis. 2nd Conf. Microbiol., Cairo.

E1-Sayed, M. Some factors affecting propagation of certain N -fixing blue-green algae.
1973 M Sc Thesis, Fac. of Agric., Cairo Univ., Egypt.[2]

El-Sayed, M. Studies on nitrogen-fixing blue-green algae in Egyptian soils. Ph D Thesis,
1978 Fac. of Agric. Ain Shams Univ. Egypt.

Espinas, C.R., and Watanabe, I. Potentiality of nitrogen-fixing Azolla-Anabaena complex as
1976 fertilizer in paddy soil. IRRI, Saturday Seminar, August 14, 1976.

FAO. China: recycling of organic wastes in agriculture. FAO Soils Bulletin 40. Rome.
1978

Fahraeus, G., and Ljunggren, H. The possible significance of pectic enzymes in root hair-
1959 infection by nodule bacteria. Physiol Plant. 12:145-154.

Fay, P. Cell differentiation and pigment composition in Anabaena cylindrica. Arch.
1969 Microbiol. 67:62-70.

Fay, P., and Fogg, G.E. Studies on nitrogen-fixation by blue-green algae, III. Growth
1962 and nitrogen-fixation in Chlorogloes fritschii mitra. Arch. Microbiol. 42:310-321.

Fay, P., and Kulasooriya, S.A. Tetrazolium reduction and nitrogenase activity in
1972 heterocystous blue-green algae. Arch. Microbiol. 87:341-352.

Felfoldy, L.M., and Zssuzsa, F.K. Some methodical observations on the use of antibiotics
1959 for preparing bacteria-free algal cultures. Act. Biol. Acad. Scient. Hung. 10 (1):95.

Fogg, G.E. Growth and heterocyst production in Anabaena cylindrica Lemm. II. In relation
1949 to carbon and nitrogen metabolism. Ann.Bot. N.S. 13:241-259.

Fogg, G.E., and Than-Tun. Interrelations of photosynthesis and assimilation of elementary
1960 nitrogen in blue-green alga. Proc. R. Soc. B. 153:111-127.

Fogg, G.E., Stewart, W.D.P., Fay, P., and Walsby, A.E. The blue-green algae. Academic
1973 Press, London, England.

Foulds, W. Effect of drought on three species of Rhizobium. Pl. Soil. 35:665-667.
1971

Fraser, M.E. Pre-inoculation of lucerne seed.J. Appl. Bact. 29:587-595.
1966

Fraser, M.E. A method of culturing Rhizobium meliloti on porous granules to form a pre-
1975 inoculant for lucerne seed. J. Appl. Bact. 39:345-351.

Fred, E.B., Baldwin, I.R., and McCoy, E. Root nodule bacteria and leguminous plants.
1932 University of Wisconsin. Studies No. 5, Madison.

Fujiware, A., and Okutsu, M. Cultural and physiological studies of the N-fixing blue-green
1959 alga, Nostoc spongiaeforme. II. The influence of the changes of hydrogen ion concentration, illumination and aeration on the growth of the alga. Tohoku Jour. Agric. Res. 10:321-325.

Gargantini, H., and Wutke, A.C.P. Atmospheric nitrogen-fixation by bacteria associated
1960 with roots of bean and cowpea. Bragantia. 19:639-652.

Gerloff, G.C. The comparative boron nutrition of several green and blue-green algae.
1968 Physiol. Plant. 21:369-377.

Gerloff, G.C., Fitzgerald,G.P., and Skoog, F. The isolation, purification and culture
1950 of blue-green algae. Am. J. Bot. 37:216-218.

Golterman, H., and Cylmo, R.S. Methods for chemical analysis of fresh waters.
1968

Gopal, B. Contribution of Azolla Pinnata R. Bl to the productivity of temporary ponds at
1967 Varanasi. Trop. Ecol. 8:126-130.

Graham, P.H. A study on the application of serodiagnostic and computative methods of the
1963 a taxonomy of the root-nodule bacteria of legumes. Ph D Thesis Univ. West Australia.

Graham, P.H. Vitamin requirements of root-nodule bacteria. J. Gen. Microbiol. 30:245-248.
1963 b

Graham, P.H., and Parker, C.A. Diagnostic features in the characterization of the root-
1964 nodule bacteria of legumes. Pl. Soil. 20:383-396.

Granhall, M. Acetylene reduction by blue-green algae isolated from Swedish soils.
1970 Oikos. 21:330-332.

Granhall, M. Nitrogen-fixation by blue-green algae in temperate soils. In: Nitrogen-
1976 fixation by free-living microorganisms. (ed. W.D.P. Stewart). Cambridge University Press. 189-197

Gupta, U.S., Maheshwari, K.L., and Sen Gupta, S.R. A method for obtaining algal cultures
1956 free from associated microorganisms. Curr. Sci. 25 (3):94.

Hallor, W.T., Sutton, D.L., and Burlow, W.C. Effect of salinity on the growth of several
1974 aquatic macrophytes. Ecology. 55:891-894.

Ham, G.E., Lawn, R.J., and Brun, W.A. Influence of inoculation, nitrogen fertilizers and
1975 photosynthetic source - sink manipulations on field grown soybeans. In: Symbiotic nitrogen-fixation in plants. (ed. P.S. Nutman). Cambridge Univ. Press. 239-253.

Hamdi, Y.A. Effect of combinations of some sulphur-containing amino acids on the efficiency
1968 of Rhizobium meliloti strains. Arch. Microbiol. 63:227-231.

Hamdi, Y.A. Stability of D-methionine-adapted strains of R. meliloti. Acta Microbiol.
1969 Polon. 18:85-86.

Hamdi, Y.A. Soil water tension and the movement of rhizobia. Soil Biol. Biochem.
1971 3:121-126.

Hamdi, Y.A., and Al-Tai, A.M. Salt tolerance of strains of R. meliloti and R. trifolii
1976 to the chlorides of sodium, calcium and magnesium. SOSLR, Technical Bull. 9:19.

Hamdi, Y.A., El-Nawawy, A.S., and Tewfik, M.S. Effect of herbicides on growth and nitrogen
1970 fixation by the alga Tolypothrix tenuis. Acta Microbiol. Polon. 2:53-56.

Hamissa, M.R., Khadr, M.S., and Gad, A.Y. The fertilizing value of blue-green algae as a
1978 N-fixer for paddy field in Egypt. Agric. Res. Rev., Egypt. (In Press).

Hardy, R.W.F., Burns, R.C., and Holsten, R.D. Application of the acetylene ethylene
1973 reduction assay for measurement of nitrogen-fixation. Soil Biol. Biochem.
5:47-81.

Hardy, R.W.F., and Havelka, U.D. Symbiotic N_2 fixation. Multifold enhancement by CO_2
1973 enrichment on field-grown soybeans. Pl. Physiol. Baltimore 51: Suppl. 35.

Hastings, A., and Drake, D. Further tests to improve inoculation of clovers. N.Z.J. Agric.
1972 125 (3):53-55.

Haystead, A., Robinson, R., and Stewart, W.D.P. Nitrogenase activity in extracts of
1970 heterocystous and non-heterocystous blue-green algae. Arch. Microbiol.
75:235-243.

Hegazi, N.A., and Niemela, S.I. A note on the estimation of Azotobacter densities by
1976 membrane filter technique. J. Appl. Bacteriol. 41:311-313.

Hegazi, N.A., Edi, M., Farag, R.S., and Monib, M. Asymbiotic N_2 - fixation in the rhizosphere
1979 of sugar cane planted under semi-arid conditions of Egypt. Rev. Ecol. Biol. Sci.
l6 (1):23-37.

Hely, F.W., Hutchings, R.J., and Zorin, M. Legume inoculation by spraying suspensions of
1976 nodule bacteria into soil beneath seed. J. Aust. Inst. Agric. Sci.
42:241-244.

Henriksson, E., Englund, B., Heden, M.B., and Was, I. Nitrogen-fixation in Swedish soils
1972 by blue-green algae. Ist. Intal. Symp. on Taxonomy & Biology of Blue-Green
Algae, Madras, Jan. 8-13, 1970.

Hill, D.J. The pattern of development of Anabaena in Azolla-Anabaena symbiosis. Plants.
1975 122:178-184.

Hill, D.J. The role of Anabaena in the Azolla-Anabaena symbiosis. New Phytol. 78:611-616.
1977

Holding, A.J., and King, J. The effectiveness of indigenous populations of R. trifolii in
1963 relation to soil factors. Plant, Soil. 18(2):191-198.

Holl, F.B., and La Rue, T.A. Host genetics and nitrogen-fixation. In: World soybean
1976 research. (ed. L.D. Hill). Proc. of the World Soybean Res. Confr.

Holland, A.A., Parker, C.A. Studies on microbiol antagonism in the establishment of
1966 clover pasture, II. The effect of saprophytic soil fungi upon Rhizobium
trifolii and the growth of subterranean clover. Pl. Soil 25:329-340.

Holm-Hansen, 0., Gerloff, G.C., and Skoog, F. Cobalt as an element for blue-green algae.
1954 Physiol-Plantarum 7:665-675.

Holst, R.W. Studies of the growth and nitrogen metabolism of the Azolla mexicana-Anabaena
1976 azollae symbiosis. Ph D Dissertation, Southern Illinois Univ. at Carbondale.

Holsten, R.D., Burns, R.C., Hardy, R.W.F., and Herbert, R.R. Establishment of symbiosis
1971 between Rhizobium and plant cells in vitro. Nature. 232 (5307):173-176.

Hoogenhout, H., and Ameoz, T. Arch. Microbiol. (Cited from Venkataraman, 1969). 50:10
1965

Horne, A.J. The ecology of nitrogen-fixation on Signy Island, South Orkney Islands. Brit.
1972 Antarctic. Surv. Bull. 27:1-18.

Hosoda, K., and Takata, H. Effect of nitrogen-fixing blue-green algae Tolypothrix tenuis
1955 on the growth of rice plants (in Japanese). Trans. Soc. Agri. Sci. 10(4):l-15.

Ibrahim, A.N., Kamel, M., and El-Sherbiny, M. Effect of inoculation with alga Tolypothrix
1971 tenuis on the yield of rice and soil nitrogen balance. Agrok. Talajtan,
20:389-400.

IRRI. Nitrogen and Rice (Symposium 18-21 September 1978). International Rice Research
1979 Institute, Los Banos, Laguna, Philippines. 499 p.

Ismunadji, M., Zulkarnaini, I., Prawirosanudro, A., and Yazawa, F. Productivity of some
1973 major Java soils with special reference to yield and nitrogen nutrition of
lowland rice. Contrib. Cent. Res. Inst. Agric. Bogor 7:1-17.

Iswaran, V., Sundara Rao, W.V.B., Magu, S.P., and Jewhri, K.S. Indian peat as a carrier
1969 of Rhizobium. Curr. Sci. 38:468-469.

Jha, K.K., Ali, M.A., Singh, R., and Bhattacharya, P.B. Increasing rice production through
1965 the introduction of Tolypothrix tenuis, a nitrogen-fixing blue-green alga.
J. Indian Soil Sci. 13:161-166.

Johnson, C.V., Mayeux, P.A., and Evans, H.J. A cobalt requirement for symbiotic growth of
1966 Azolla filiculoides in the absence of combined nitrogen. Plant Physiol.
41:852-855.

Jordan, C., and Allen, O.N. Genus I. Rhizobium. In: Bergeys' Manual of determinative
1974 bacteriology. (eds. R.E. Buchanan and N.E. Gibbons). The Williams and
Wilkins Co., Baltimore. 262-265.

Kang, B.T., Nangju, D., and Ayanaba, A. Effect of fertilizer use on cowpea and soybean
1975 nodulation and nitrogen-fixation in the lowland tropics. In: Biological
nitrogen-fixation systems in the tropics. (eds. A. Ayanaba and P.J. Dart).
John Wiley and Sons. 205-216.

Keya, W.C.O., and Van Eijnatten, C.L.M. Studies on oversowing of natural grassland in
1975 Kenya. 2. Effects of seed inoculation and pelleting on the nodulation and
productivity of Desmodium uncinatum. East Afr. Agric. For. J.
40:351-158.

Kleczkowska, J. Genetical changes in Rhizobium bacteria and their bacteriophages during
1971 coexistence. Plant and Soil special volume·47-56.

Khadr, M.S. Studies on nitrogen-fixing blue-green algae in Egyptian soils. Ph D Thesis,
1975 Fac. Agric. Al-Azhar University, Cairo.

Khadr, M.S., Hamissa, M.R., and Gad, A.Y. Response of paddy to nitrogen-fixing blue-green
1978 algae. Agric. Res. Rev. Egypt (In Press).

Kiyohara, T., Fugita, Y., Hattori, A., and Watanabe, A. Effect of light on glucose
1962 assimilation in Tolypothrix tenuis J. Gen. Appl. Microbiol. 8:165-168.

Koyama, T., Chammek, C., and Niamscrihand. Soil plant nutrition studies on tropical rice,
1971 III. The effect of soil fertility status of nitrogen and its liberation upon the nitrogen fertilization of rice plants in Bangkhen paddy soil. Soil Sci. Plant Nutrition. 17:210.

Kratz, W.A., and Mayers, J. Photosynthesis and respiration of three blue-green algae.
1955 Pl. Physiol. 30:275-280.

Kraus, M.P. Preparation of pure blue-green algae. Nature, 211 (5045):310.
1966

Krieg, N.R. Taxonomic Studies of Spirillum lipoferum. In: Genetic engineering for
1977 nitrogen-fixation. (ed. A. Hollaender). Plenum Press, New York and London. 463-470.

Kulasooriya, S.A., and de Silva, R.S.Y. Effect of Azolla on yield of rice. IRRI.
1977 Newsletter 2 (3):10.

La Rue, T.A. The bacteria. In: A treatise on dinitrogen fixation, section III, Biology
1977 (eds. R.W.F. Hardy and W.S. Silver). Wiley-Interscience Pub., London, Sydney, Toronto. 19-63.

Lawrence, D.B. Glaciers and vegetation in south eastern Alaska. Am. Scient. 46:89-122.
1958

Ley, S.H. The effect of nitrogen-fixing blue-green algae on the yields of rice plant.
1959 Acta Hydrobiol. Sinica 4:440-444.

Lorkiewicz, Z., Zurkowski, W., Kowalczuk, E., and Gorska-Melke, A. Mutagenesis and conjugation in R. trifolii . Acta Microbiol. Polon. Ser A. 3:101-107.

Lynn, R.L., and Vogelsberg, M.C. The role of algae in crust formation and nitrogen
1974 cycling in desert soils. US/IBP Desert Biome Research. Memo Random 74-41 (May 74).

Lumpkin, T.A., and Plucknett, D.L. Azolla: botany, physiology and use as green manure.
1978 Hawaii Agric. Exp. Station J. No. 2294.

Mathews, L.J. Weed identification and control: Azolla rubra. N.Z.J. Agric. 106:279.
1963

Miguel, C., and Rodriguez-Barrueco, C. Acetylene reducing activity of detached root
1974 nodules of Myrico Faya Aif. Plant and Soil. 41:521-526.

Moore, A.W. Azolla: biology and agronomic significance. Bot. Rev. 35:17-34.
1969

Moustafa, M.A., and Mahmoud, M.Z. Bacterial isolates from root nodules of Zygophyllaceae.
1951 Nature. (Lond). 167:446-447.

Mulder, E.G., and Van Veen, W.L. Effect of pH and organic compounds on nitrogen-fixation
1960 by red clover. Plant and Soil. 13:91-113.

Neyra, C.A., Dobereiner, J. Nitrogen-fixation in grasses. In: Advances in agronomy.
1977 (ed. N.C. Brady) Vol. 24 Academic Press, London and New York.

Newton, J.W. Photo-production of molecular hydrogen by a plant algal symbiotic system.
1976 Science. 169:559-560.

Newton, J.W., and Herman, A. Isolation of a blue-green alga from Azolla. Plant Physiol.
1977 59:5-514.

Nishigaki, S., Shibuya, M., and Kodaira, K. (Cited from A. Watanabe, 1965).
1953

Nutman, P.S. The relation between root hair infection by Rhizobium and nodulation in
1962 Trifolium and Vicia. Proc. Roy. Soc. B. 156:122-137.

Nutman, P.S. Perspectives in biological nitrogen-fixation. Sci. Prog. Oxf. 59:55-74.
1971

Nutman, P.S. Rhizobium in soil. In: Essays in soil microbiology. (ed. H. Walker).
1975 Butterworths Sci. Pub. 111-131.

Nutman, P.S. IBP field experiments on nitrogen-fixation by nodulated legumes. In: Nitrogen
1976 fixation in plants. (ed. P.S. Nutman). Cambridge University Press. 211-237.

Obaton, M. Utilization de mutants Spontanes resistants aux antibiotiques pour 1'etude
1971 ecologique du Rhizobium C.R. Acad. Sci. Ser. D. 272:2630-33.

Ohmori, K., and Hattori, A. Induction of nitrate and nitrate reductases in Anabaena
1970 cylindrica. Pl. Cell Physiol. Tokyo 11:873-878.

Okon, Y., Albrecht, S.L.,and Burris, R.H. Methods for growing S. lipoferum and for
1977 counting it in pure culture and association with plants. App. Environ.
Microbiol. 33(1):85-88.

Olsen, G. On biological nitrogen-fixation in nature, particularly in blue-green algae.
1972 C.r. Trav. Lab. Carlsberg. 37:269-283.

Oosthuizen, G.J., and Walters, M.M. Control water fern with diesoline. Farming South
1961 Africa. 37:35-37.

Orellana, R.G., Sloger, C., and Miller, V.L. Rhizoctonia-Rhizobium interactions in relation
1976 to yield parameters of soybean. Phytopathol. 66(4):464-472.

Pagan, J.D., Child, J.J., Scowcroft, W.R., and Gibson, A.H. Nitrogenase activity in
1975 rhizobia in absence of plant host. Nature (London) 256:406-407.

Patel, J.J. Microorganisms in the rhizosphere of plants inoculated with Azotobacter
1969 chroococcum. Plant and Soil. 31(2):209-223.

Peters, G.A. Studies of the Azolla-Anabaena azollae symbioses. In: W.E. Newton and
1976 C.J. Nyman, eds. Proceedings of the lst international symposium on
nitrogen-fixation. Vo. 2, Pullman, Washington, U.S. 1974. Washington
State University Press. 592-610.

Peters, G.A. The AzollZa-Anabaena azollae symbioses. In: Genetic engineering for nitrogen-
1977 fixation. (ed. A. Hollaender). Plenum Pub. Co. New York. 231-259.

Peters, G.A. Studies on the Azolla-Anabaena azollae symbioses. C.F. Kettering Res. Lab.
1978 Yellow Springs, Ohio. Contribution No. 518.

Peters, G.A., and Mayne, B.C. The Azolla-Anabaena azollae relationship I. Initial
1974 characterization of the association. Plant Physiol. Lancaster. 53:813-819.

Peters, G.A., Mayne, B.C., Ray, T.B., and Toia, R.E. Physiology and biochemistry of the
1979 Azolla-Anabaena symbionts. Symposium on nitrogen and rice. 18-21 September 1978. IRRI Los Banos, Philippines.

Peters, G.A., Toia, R.E., Jr., Levine, N.J., and Raveed, D. The Azolla-Anabaena azollae
1978 relationship. VI. Morphological aspects of the association. New Phytol. 80:583-593.

Peters, G.A., Evans, W.R., and Toia, R.E., Jr. The Azolla-Anabaena azollae relationship.
1976 4. Photosynthetically driven, nitrogenase-catalysed H_2 production. Plant Physiol. Lancaster. 58:119-126.

Pieterse, A.H., de Lange, L., and Van Vliet, J.P. A comparative study of Azolla in the
1977 Netherlands. Acta Bot. Neerl. 26:433-449.

Pilai, R.N., and Sen, A. Salt tolerance of R. trifolii . Indian J. Agric. Sci. 36:74.
1966

Pimental, D. Food, nitrogen and energy. In: Proc. Ist. Int. Symp. Nitrogen-Fixation,
1976 Pullman. (eds. W.E. Newton and C.J. Nyman). Washington State University Press. 656-673.

Quispel, A. Prerequisites for biological nitrogen-fixation in root nodule symbiosis.
1974 In: The biology of nitrogen-fixation. (ed. A. Quispel). Amsterdam, North Holland. 719-745.

Rabb, J.L., Willis, L.D., and Dunigan, E.P. Soybean inoculation research. Ann. Res. Rept.
1976 Red River Valley Agr. Exp. Stn. Louisiana. 116-117.

Raicheva, L. Agar and peat inoculation efficiency in Bulgaria. In: P.S. Nutman. Symbiotic
1976 nitrogen-fixation in plants. Cambridge University Press. 199-202.

Rains, D.W., and Talley, S.N. The use of Azolla as a source of nitrogen for temperate
1978 zone rice culture. Proc. IMPACT Program East West Centre.

Relwani, L.L., and Subrahmanyan, R. Role of blue-green algae, chemical nutrients and
1963 partial soil sterilization on paddy yield. Curr. Sci. 32:441-443.

Ridge, E.H., and Rovira, A.D. Microbiol inoculation of wheat. 9th Intern. Congr. of
1968 Soil Sci. Trans. Vol. III. 473-481.

Rinaudo, G., Hamad-Fares, I., and Dommergues, Y.R. Nitrogen-fixation in the rice
1975 rhizosphere:methods of measurement and practices suggested to enhance the process. In: Biological nitrogen-fixation in farming systems of the tropics. (eds. A. Ayanaba and P. Dart). J. Wiley and Sons. 313-322.

Rizk, S.G. Atmospheric nitrogen fixed by legumes under Egyptian conditions, Egyptian
1962 clover (Trifolium alexandrinum). J. Soil Sci. UAR 2:253-70.

Rizk, S.G. Significance of legumes in nitrogen-fixation under Egyptian conditions.
1968 El-Felaha 5:438-449. (In Arabic).

Robinson, P.E. Root knot nematodes and legume nodules. Nature (Lond) 189:506.
1961

Roughley, R.J. The preparation and use of legume seed inoculants. Plant and Soil,32:675-
1970 701.

Roughley, R.J., and Vincent, J.M. Growth and survival of Rhizobium spp in peat culture.
1967 J. App. Bacteriol. 30:362-76.

Roughley, R.J., Date, R.A., and Walker, M.H. Inoculating and lime pelleting legume seeds.
1966 Agric. Gaz. N.S. Wales 77 (Pt.4).142-146.

Rubenchick, L.I. Azotobacter and its use in agriculture. Translated from Russian.
1960 Published for the National Science Foundation, Washington D.C. US Dept. of Commerce, Washington 25, D.C.

Ruinen, J. Nitrogen-fixation in the phyllosphere. In: Nitrogen-fixation by free-living
1975 microorganisms. (ed. W.D.P. Stewart). Cambridge Univ. Press. 85-100.

Ruschel, A.P., and Vose. Present situation concerning studies on associative nitrogen-
1977 fixation in sugarcane, Saccharum officinarum L. CENA Scientific Bulletin 45, Brazil.

Ruschel, A.P., Filho, J.O., Junior, E.Z., and Henis, Y. Aerobic and anaerobic nitrogen-
1977 fixing bacteria on sugarcane roots. Proc. The XVI Congress of Soc. of Sugar-cane Technologists. Sao Paulo, Brazil, Vol:2:1923-1929.

Russell, E.J. Soil conditions and plant growth 9th ed. John Wiley and Sons, New York.
1961

Sabet, Y.S. Bacterial root nodules in the Zygophyllaceae. Nature, Lond. 157:656.
1946

Sahay, M.N. Nitrogen uptake by the rice crop in the experiments with blue-green algae.
1966 Proc. Indian Acad. Sci. Sect. B 638:223-233.

Salem, S.H., and Szegi., J. Growth and survival of Rhizobium phaseoli at different
1971 temperatures in some Hungarian soil types. Agrok. Talajtan.20:361-368.

Sampaio, M.J.A., de Vasconclos, L., and Dobereiner, J. Characterization of three groups
1978 within Spirillum lipoferum Beijerinck. Ecol. Bull. (Stockholm).26:364-365.

Sankaram, A. Blue-green algae. Role in rice culture. Farmer and Parliament. 11:9-10.
1967

Sankaram, A. Work done on blue-green algae in relation to agriculture. Bull. No. 27,
1971 Indian Council of Agric. Res., New Delhi.

Saubert, G.G.P. Provisional communication on the fixation of elementary nitrogen by a
1949 floating fern. Ann. Royal Botanic Gardens. Buitenzorg. 51:177-197.

Saxena, M.C., and Tilak, V.B.R. Response to inoculation in soybean and its residual effect
1975 on wheat. Ind. J. Agron. 20(4):369-370.

Serry, A., Hamissa, M.R., Shoukry,A., Mishrik, E.S., and Abd el-Aziz, M.S. Studies on the
1970 nitrogen nutrition of paddy. Rice Res. Committee Jan. 24-26 1970, Cairo, Egypt.

Schank, N.C., and Hinson, K. Response of nodulating and non-nodulating soybeans to a species
1978 of Endogone mycorrhiza, Agron. J. 65:849-850.

Schmidt, E.L. Legume symbiosis: A. Ecology of the legume root bacteria. In: Interactions
1978 between non-pathogenic soil microorganisms and plants. (eds. Y.R. Dommergues and S.V. Krupa). Elsevier, Amsterdam, Oxford. 269-303.

Van Schrevan, D.A. Methods used in the Netherlands for the production of legume inoculants
1958 In: Nutrition of legumes (ed. E.G. Hallsworth). Butterworth and Co., London. 328-333.

Van Schrevan, D.A. Some factors affecting growth and survival of Rhizobium spp in soil
1970 peat cultures. Plant and Soil. 32:113-130.

Schwinghamer, F.A. Genetic aspects of nodulation and dinitrogen fixation by legumes:
1977 The microsymbiont. In: A treatise on dinitrogen fixation. Section III Biology. (eds. R.W.F. Hardy and W.S. Silver). A Wiley-Interscience Publication, London, Sydney, Toronto. 577-622.

Shalan, N.S. Comparative studies on nitrogen-fixation by local and imported strains of
1974 blue-green algae. M Sc Thesis, Fac. of Agriculture, Ain Shams University, Egypt.

Shen, C., Lu, S., Chen, K., and Ge, S. The initial experiment of Azolla's nitrogen-fixing
1963 ability. Pedology Bull. Peking, Bimonthly 4 (8):46-48.

Shtina, E.A. The development of soil algae in podzolic soils. Pochrovedenia 1957, 12.
1957

Shtina, E.A., Pankratova, F.M., Perminova, G.N., Tretyakova, A.N., and Yung, L.A.
1968 Distribution and the role of N-fixing blue-green algae in the soils of the temperate zone of U.S.S.R. Trans. Inst. Congr. Soil Sci. 9th. 2:151-181.

Singh, P.K. Azolla plants as fertilizer and feed. Indian Farming, 27:19-22.
1977

Singh, P.K. Nitrogen economy of rice soils in relation to nitrogen-fixation by blue-green
1978 algae and Azolla. In: Symposium on increasing rice yield in Kharif held at CRRI, Cuttack, 1978.

Singh, P.K. Use of Azolla in rice production in India. Symposium on Nitrogen and Rice
1979 18-21 September 1978. IRRI, Los Banos, Philippines.

Singh, P.K., and Subudhi, B.P.R. Utilize Azolla in poultry feed. Indian farming.27:37-38.
1978

Singh, R.N. The fixation of elementary nitrogen by some of the commonest blue-green algae
1942 from the paddy field. Soils of the United Provinces of Bihar. Ind. J. Agric. Sci. 2743-756.

Singh, R.N. Role of blue-green algae in N-economy of Indian agriculture. Indian Council
1961 of Agric. Res., New Delhi.

Singh, R.N., and Singh, P.K. Isolation of cyanophage from India. Nature, Lond. 216:1020.
1967

Smith, R.L., Bouton, J.H., Schank, S.C., and Quesenberry, K.H. Yield increases of tropical
1975 grain and forage grasses after inoculation with S. lipoferum in Florida. In: Biological nitrogen-fixation in farming systems of the tropics (eds. A. Ayanaba and P.J. Dart). J. Wiley and Sons. New York, Brisbane, Toronto. 307-311.

Smith, R.L., Schank, S.C., Bouton, J.H., and Quesenberry, K.H. Yield increases of tropical
1978 grasses after inoculation with Spirillum lipoferum. Ecol. Bull. (Stockholm). 26:380-385.

Soeder, C.J. The technical production of micro-algae and its prospects in marine
1976 aquaculture. In: Harvesting polluted waters (ed. 0. Devik). Plenum Press, New York. 11-26.

Solorzano, L. Determination of ammonia in natural waters by the phenolhypochlorite method.
1969 Limnol. Oceanogo. 14:799-801.

Sprent, J. Nitrogen-fixation by legumes subjected to water stress and light stress.
1976 In: Symbiotic nitrogen-fixation in plants. (ed. P.S. Nutman). Cambridge University Press, London. 405-420.

Stanier, R.Y. Division I. The cyanobacteria. In: Bergeys Manual of determinative
1974 bacteriology (eds. R.E. Buchanan and N.E. Gibbons). The Williams Wilkins Co., Baltimore. 22.

Staub, R. Ernahrungsphysiologische autokologische Untersuchungen an der planktischen
1961 Blaualge Oscillatoria rubescano DC. Schweiz. Z. Hydrol. 23:82-198.

Steinborn, J., and Roughley, R.J. Sodium chloride as a cause of low number of Rhizobium
1974 in legume inoculants. J. Appl. Bacteriol. 37:93.

Stewart, W.D.P. Nitrogen turnover in marine and brackish habitats. l. Nitrogen-fixation
1965 Ann. Bot. 20:229-239.

Stewart, W.D.P. Transfer of biologically fixed nitrogen in a sand dune slack region.
1967 Nature, Lond. 214:603-604.

Stewart, W.D.P. Nitrogen-fixation by photosynthetic microorganisms. Ann. Rev. Microbiol.
1973 27:283-316.

Stewart, W.D.P. Biological cycling of nitrogen in intertidal and supralittoral marine
1975 environment. In Proc. 9th European Marine Biol. Symposium (ed. H. Barnes) Aberdeen Univ. Press. 637-660.

Stewart, W.D.P. Nitrogen-fixation Phil. Trans. R. Soc. Lond. B274:341-358.
1976

Stewart, W.D.P. Blue-green algae. In: A treatise on dinitrogen fixation,section III
1977 biology. (eds. R.W.F. Hardy and W.S. Silver). John Wiley and Sons. Interscience Pub. New York, London. 63-123.

Stewart, W.D.P., Haystead, A., and Pearson, H.W. Nitrogenase activity in heterocysts of
1969 blue-green algae. Nature, Lond. 224:226-228.

Stewart, W.D.P., and Lex, M. Nitrogenase activity in the blue-green alga Plectonema
1970 boryanum strain 594. Arch. Microbiol. 73:250-260.

Stewart, W.D.P., and Pearson, M.C. Nodulation and nitrogen-fixation by Hippophae rhamhoides
1967 L. in the field. Plant and Soil. 26:348-360.

Stewart, W.D.P., and Rowell, P. Effects of L-methionine DL-sulphoximine on the assimilation
1975 of newly fixed NH_3, acetylene reduction and heterocyst formation in Anabaena cylindrica. Biochem. Biophys. Res. Commun. 65:846-856.

Strijdom, B.W., and Deschodt, C.C. Carriers of rhizobia and the effects of prior treatment
1976 on the survival of rhizobia. In: Symbiotic nitrogen-fixation. (ed. P.S. Nutrnan) Cambridge University Press. 151-168.

Subba Rao, N.S. A case for production of bacterial fertilizers application. In: Symbiotic
1976 nitrogen-fixation in plant IBP. No. 7. (ed. P.S. Nutman). Cambridge University Press. 255-268.

Subba Rao, N.S., and Balasundaram, V.R. Rhizobium inoculants for soybean. Indian Farming
1971 21:22-23.

Subrahmanyan, R. Some observations on the utilization of blue-green algae mixtures in rice
1972 cultivation in India. In: Taxonomy and biology of the blue-green algae. (ed. T.V. Deskachary). Madras University Press, Madras. 281-293.

Subrahmanyan, R., and Sahay, M.N. Observations on nitrogen-fixation by some blue-green
1964 algae and remarks on its potentialities in rice culture. Indian Acad. Sci. Proc., Sect. B,60:145-154.

Subrahmanyan, R., Relwani, L.L., and Patnaik, S. Preliminary observations on the
1965 interaction of different rice soil types to inoculation of blue-green algae in relation to rice cultures. Proc. Indian Acad. Sci. Sect. B,62:171-175.

Subudhi, B.P.R., and Singh, P.K. Nutritive value of water fern Azolla for chicks. Poult.
1978 a Sci., 57:378-380.

Subudhi, B.P.R., and Singh, P.K. Effect of macronutrients and pH on growth, nitrogen-
1978 b fixation, and soluble sugar content of water fern Azolla pinnata. Biol. Plant. (In press).

Subudhi, B.P.R., and Singh, P.K. Heterocyst spacing in the symbiotic blue-green algae
1978 c Anabaena azolla. Curr. Sci., 49:510-511.

Sundara Rao, W.V.B., Goyal, S.K., and Venkataraman, G.S. Effect of inoculation of Aulosira
1963 fertilissima in rice plants. Curr. Sci. 32:366-367.

Taha, M.S. Isolation of some nitrogen-fixing blue-green algae from the rice fields of
1963 Egypt in pure culture. Microbiologiya, USSR. 32:421-425.

Taha, M.S. Effect of nitrogen compounds on the growth of blue-green algae and fixation of
1964 molecular oxygen by them. Microbiologiya, USSR. 33:397-403.

Taha, E.E.M., and El-Refai, A.E.H. Physiological and biochemical studies on nitrogen-
1962 fixing blue-green algae. II. The role of calcium, strontium, cobalt and molybdenum in nitrogen-fixation of Nostoc commune. Arch. Microbiol. 43:67—75.

Taha, S.M., El-Nawawy, A.S., Abd el-Hafez, A.M., El- Sawy, A.M. and El-Borollosy, M.A.
1973 Effect of inoculation with blue-green algae on rice growth and nitrogen-fixation: Ain Shams Res. Bull. 54:3-15.

Taha, S.N., El-Nawawy, A.S., Abd el-Hafez, A.M., El-Sawy, M., and El-Borollosy, M.A. Effect
1972 of inoculation with blue-green algae on rice growth and N-fixation. Fac. Agric. Ain Shams Res. Bull. 54:1-4.

Taha, S.M., E1-Nawawy, A.S., Abd el-Hafez, A.M., El-Sawy, M., and El-Borollosy, M.A. Growth
1974 and nitrogen-fixation capacity of a local isolate of the blue-green alga Nostoc linkia var. arvense. J. Microbiol (ARE) 9:(1 & 2) 119-128.

Taha, S.M., Mahmoud, S.Z., and Salem, S.H. Effect of inoculation with rhizobia on some
1967 leguminous plants in UAR. II. Nitrogen Fertilization J. Microbiol, ARF.2:31-41.

Talley, S.N., Talley, B.J., and Rains, D.W. Nitrogen-fixation by Azolla in rice fields.
1977 In:Genetic engineering in nitrogen-fixation. (ed. A. Hollaender). Plenum Press, New York. 259-281.

Tarrand, J., Krieg, W., and Dobereiner, J. A taxonomic study of the Spirillum lipoferum
1978 group, with descriptions of a new genus, Azospirillum gen. nov. and two species, Azospirillum lipoferum (Beijerinck) comb. nov. and Azospirillum brasilense sp nov. Ban. J. Microbiol. 24:967-980.

Ter Meulen, H. Sur 1'accumulation du molybdene chez quelques plantes aquatiques. Recl.
1932 Trav. Chim. Pays-Bas. 51:549-550.

Thuyet, T.Q., and Tuan, D.T. Azolla: a green manure. Agric. Problems. Vietnamese Studies.
1973 38:119-127.

Thompson, J.A., Brockwell, J., and Roughley, R.J. Pre-inoculation of legume seed. J. Aust.
1975 Inst. Agric. Sci. 41:253-254.

Trinick, M.J. Symbiosis between Rhizobium and the non-legume, Trema aspera. Nature,
1973 London. 244:459-60.

Trukhin, N.V. Optimal pH values for the growth of certain blue-green algae. Biol. Abst.
1960 36:42312.

Tomya, H. Ann. Rev. Plant Physiol. (Cited from Venkataraman, 1969). 8:309.
1957

Tuan, D.T., and Thuyet, T.Q. Use of Azolla in rice production in Vietnam. Symposium on
1979 nitrogen and rice. 18-21 September 1978. IRRI, Los Banos, Philippines.

Vaidya, B.S., Patel, I.M., and Jani, V.M. Secretion of a highly reducing substance by
1970 algae in media and its possible role in crop physiology. Sci. Culture. 37: 383-384.

Vancura, V. Detection of gibberellic acid in Azotobacter cultures. Nature, Lond.
1961 192:88-89.

Venkataraman, G.S. The role of blue-green algae in agriculture. Science and Culture Vol.
1961 27, No. 1.

Venkataraman, G.S. Studies on nitrogen-fixation by blue-green algae. III. Nitrogen
1962 fixation by Anabaena azollae. Indian J. Agric. Sci. 32:22-24.

Venkataraman, G.S. The cultivation of algae. Indian Council for Agric. Res., New Delhi.
1969

Venkataraman, G.S. Alga biofertilizers and rice cultivation. Today and Tomorrow's
1972 Printers and Pubs. Faridshad, Hanyana, 75 p.

Venkataraman, G.S. Algae inoculation of rice fields. Paper presented at the Nitrogen and
1978 Rice Symposium, IRRI, Los Banos, Laguna, Philippines. Sept. 18-21, 1978.

Venkataraman, G.S., and Rajyalakshimi, B. Relative tolerance of blue-green algae to
1971 pesticides. Indian Agric. Sci., 42:119-121.

Venkataraman, G.S., and Rajyalakshimi, B. Relative tolerance of blue-green algae to
1972 pesticides. Indian J. Agric. Sci. 42:119.

Venkataraman, G.S., Jacob, K.M., and Goyal, S.K. Nitrogen-fixation by the endophytic alga
1964 from the coralloid roots of Cycas revoluta. Proc. Nat. Acad. Sci. India.
34B:153.

Venkataraman, G.S., and Goyal, S.K. Influence of blue-green algae inoculation on crop
1968 yields of rice plants. Soil Sci. and Plant Nutrition. 14:249-251.

Venkataraman, G.S., and Goyal, S.K. Influence of blue-green algae on the high yielding
1969 paddy variety IR-8. Sci. & Culture. 35:58-59.

Venkataraman, G.S., and Neelakanthan, S. Effect of the cellular constituents of the
1967 nitrogen-fixing blue-green alga, Cylindrospermum muscicola on the root
growth of rice seedlings. J. Gen. Appl. Microbiol. 13:53-61.

Vigue, J.T., Harper, J.E., and Peters, D.B. Nodulation of soybeans grown hydroponically
1977 on crop. Sci. 17 (1):169-172.

Vincent, J. Survival of the root nodule bacteria. In: Nutrition of the legumes. (ed.
1958 E.G. Hallsworth). Butterworth & Co. London. 108-123.

Vincent, J.M. Environmental factors in the fixation of nitrogen by the legume. In: Soil
1965 nitrogen. (eds. W.V. Bartholomew and F.E. Clark). Amer. Soc. Agron. Madison,
Wis. 384-435.

Vincent, J.M. A Manual for the Practical Study of Root Nodule Bacteria, IBP Handbook No. 15.
1970 Blackwell Sci. Publications, Oxford and Edinburgh. 125-126.

Vincent, J.M. Rhizobium: general microbiology. In: A treatise on dinitrogen fixation
1977 Section IV. Biology. (eds. R.W.F. Hardy and S. Silver). 277-366.

Virtanen, A.I. On the fixation of molecular nitrogen in nature. Commun. Inst. Forest.
1962 Fenniae 55. 22:1-11.

Watanabe, A. On the effect of atmospheric nitrogen-fixing blue-green algae on the yield
1956 of rice. Bot. Mag. Tokyo. 69:530-535.

Watanabe, A. Distribution of nitrogen-fixing blue-green algae in various areas of South
1959 East Asia. J. Gen. Appl. Microbiol.. Tokyo. 5:21-29.

Watanabe, A., Hattori, A., Fujita, Y., and Kiyohara, T. Large scale culture of blue-green
1959 alga, Tolypothrix tenuis, utilizing hot spring and natural gas as heat and .
carbon dioxide source. J. Gen. Appl. Microbiol. Tokyo. 5:51-67.

Watanabe, A. List of algal strains in collection at Institute of Applied Microbiology,
1960 University of Tokyo. J. Gen. Appl. Microbiol. 6:283-292.

Watanabe, A. Studies on the blue-green algae as green manure in Japan. Proc. Nat. Acad.
1965 Sci. India. 35A:361-369.

Watanabe, A. Some devices for preservation of algae. In: Cultures and collections of
1966 algae. (eds. A. Watanabe and A. Hattori). Jap. Soc. Plant Physiologists.
Tokyo, Japan.

Watanabe, I. Azolla utilization in rice cultures. IRRI Newsletter.2(3):10.
1977

Watanabe, A., Nishigaki, S., and Konishi, C. Effect of nitrogen-fixing blue-green algae on
1951 the growth of rice plants. Nature, Lond. 168:748-749.

Watanabe, A., and Yamamato, Y. Mass culturing, preservation and transportation of the
1970 nitrogen-fixing blue-green algae. Proc. Second Symp. on N. fixation and nitrogen cycle. Sendai, Japan.

Watanabe, I., Espinas, C.R., Berja, N.S., and Alimagno, B.V. Utilization of Azolla-Anabaena
1977 complex as a nitrogen fertilizer for rice. IRRI Research Paper Ser. No. 11. IRRI, Manila, Philippines.

Watanabe, I., Lee, K.K., Alimagno, B.V., Sato, M., del Rosavio, D.C., and de Guzman, M.R.
1977 Biological nitrogen-fixation in paddy field studies by in situ acetylene reduction assays. IRRI Res. Paper Ser. No. 3. 1-16.

Wieringa, K.T. A new method for obtaining bacteria-free cultures of blue-green algae.
1968 Antonie Leeuwenhoek. 34:54-56.

Williams, A.E., and Burris, R.H. Amer. J. Bot. 39:340. (Cited from Singh, 1961).
1952

Whitney, A.S. Contribution of forage legumes to the nitrogen economy of mixed swards.
1975 A review of relevant Hawaian research. In: Biological N. fixation systems in the tropics. (eds. A. Ayanaba and P.J. Dart). John Wiley and Sons. 89-96.

Wittman, W., Bergersen, F.J., and Kennedy, G.S. The coralloid roots of Macrozamia communis
1965 L. Johnson. Aust. J. Biol. Sci. 18:1129-34.

Yao, P.Y., and Vincent, J.M. Host specificity in the root hair curling factor of Rhizobium
1969 spp. Aust J. Biol. Scis. 22:413-23.