NATIONAL OPEN UNIVERSITY OF NIGERIA

COURSE CODE: CSP 401

COURSE TITLE: BIOTECHNOLOGY IN CROP/PEST MANAGEMENT

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CSP 401: BIOTECHNOLOGY IN CROP/PEST MANAGEMENT

COURSE OUTLINE

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MODULE 1: INTRODUCTION TO AGRICULTURAL BIOTECHNOLOGY

Unit 1.1: What is Biotechnology?

Biotechnology is not a new era of scientific endeavour: microorganisms have been used to produce food such as beer, vinegar, yoghurt and cheese for over 8 millennia. Simply put, Biotechnology is the use of living cells or microorganisms (e.g. bacteria) in the industry and technology to manufacture drugs and chemicals, break down waste, etc. More technically put, Biotechnology is an area of multidisciplinary science, involving a variety of distinct subjects, where living organisms or their useful parts are put into effective use to cater for the welfare of humanity. It may be grouped into:

(i) Conventional Biotechnology
(ii) Modern Biotechnology

Biotechnology is considered a powerful tool that can, in a quick and through manner, bring what is most lacking in agriculture. Question of safety of biotechnology products to man and the environment has been considered alongside Biotechnology programmes. In most developing countries of West and Central Africa where food is produced for instance, famine, poverty, and malnutrition remain huge constraints in rural as well as urban areas. Interestingly, though, agriculture, which makes up forty percent of export revenue and thirty-five percent of the gross domestic products of West and Central African revenue, employs seventy percent of the labour force and covers endless agro ecological lands.

Significantly, in many industrialized countries, biotechnology has contributed to progress in agriculture, while, in developing countries, it comes to add itself to the many technological tools to achieve crucial productivity and sustainability targets, to increase food production on the same land surface areas or less, with added nutritional value and projected lesser negative impact on environment. However, large-scale use of biotechnology has its own constraint: skilled human resources are limited; material and financial resources are lacking; the controversies about some agricultural biotechnologies, such as the genetic engineering and products from this new technology, remain widespread and time consuming. In any case, given to the skyrocketing rural population growth, its dependency on agricultural production

systems highly vulnerable to climatic changes, the scarcity of fertile lands per head, need to increase and improve agricultural production has become definitely very important.

The emergence of biotechnology over the last few decades has opened new doors for increased productivity not only for agriculture but also in medicine and industry. It is of particular relevance to developing countries that are confronted with an ever increasing population, food shortage, and scarcity of economic resources. Harnessing of solar energy to improve photosynthetic bacteria as well as utilization of agriculture and organic wastes to produce methane using biogas plants are also aspects of biotechnology in vogue. The same is true of biological nitrogen fixation, a prerogative of certain free living or symbiotic anaerobic and photosynthetic bacteria and algae. Biotechnology has been employed in biofuel production and in biological clean up of contaminated soil.

Applied to the economies of countries in the sub-region, biotechnology offers additional technological opportunities capable to responding to the constant demand for food and to reducing vulnerability in the agricultural sector. As a result, it contributes to income generation, improving of nutrition and preservation of natural resources and ecosystem services. It is necessary to ascertain whether biotechnologies can supply rapid, safe, cost effective solutions to the intractable biotic and abiotic constraints. The institutional and infrastructure constraints to agriculture are amenable to positive human intervention, and could facilitate rapid adoption of the yield and quality enhancing biotechnologies. Whether biotechnological solutions are employed is a matter of consumer demand and need, and the resolve of politicians and regulators to deal with these issues in a science and fact based manner with due resolve.

Further Reading

Ignacimuthu, S.J. 1995. Basic Biotechnology. Tata McGraw-Hill Publishing Company Limited, New Delhi. Second Reprint, 1996. 317pp.

Nwalozie, M. C. 2006. Added value: what biotechnology brings to agriculture. Quarterly Newsletter for research and Agricultural development in West and Central Africa. 38: 12pp.

Roy-Macauley, H. www.coraf.org

Unit 1.2. CONVENTIONAL BIOTECHNOLOGY

In the early days, biotechnologists used **living** organisms for the **manufacture** of a variety of useful materials. Whatever by-products were obtained during normal cell growth, were used by people. For example, during the normal growth of yeast cells in grape juice, sucrose is converted to ethanol and this fermented juice, containing alcohol, is used as wine. Similarly, *Penicillium notatum* and *P. chrysogenum* produce the antibiotic penicillin as a by-product of their secondary metabolism and this compound is used to fight microbial diseases. Microbial production of glycerol by yeast, acetone and butanol fermentation using *Clostridium acetobutylicum*, citric acid production by *Aspergillus niger* and Streptomycin production using *Streptomyces griseus* are some of the fields developed under conventional biotechnology.

Unit 1.3. MODERN BIOTECHNOLOGY

More recently, plant tissue culture or micro propagation has become a useful technology, involving the principle of **TOTIPOTENCY,** enabling a cell to segment into a whole plant in the proper medium. The production of biotechnology based plants, such as orchids, bamboos and a host of others has lead to export oriented industries in some developing countries like India. Rapid production of disease free clones of crops like yam, cassava, banana and plantain has been possible through tissue culture technique.

Modern biotechnology enables an organism to produce a totally new product, which the organism does not or cannot produce in its normal course of life. Since this means

we are able to engineer a new genetic potential in an organism, this technology is also called **GENETIC ENGINEERING**.

Technically put, Genetic Engineering is a technology in which a gene or genes are taken from one organism (the donor) or are synthesized *de novo* (afresh), possibly modified and are then inserted into another organism (the recipient) in an attempt to transfer a desired trait or character. The technique is also called Genetic Modification (GM), gene manipulation, recombinant DNA technology, gene splicing, bioengineering ... and many other things!

Similar cell-fusion between plants and microorganisms through protoplast fusion and transfer of nitrogen fixing nodules, specific genes from legumes to non-legumes, had led to interspecific hybridization, and the impact of these methodologies in various facets of agriculture cannot be over-emphasized. Basic techniques, which stimulate the progress of modern biotechnology include:

1. Recombinant DNA manipulation (genetic engineering)
2. Plant and animal tissue culture
3. Protoplast fusion
4. Monoclonal antibodies
5. Protein engineering
6. Immobilized enzymes and cell catalysis
7. Biosensors
8. Computer-aided bioprocess
9. New reactor design
10. DNA transfer into living cells
11. Polymerase chain reaction
12. Chromosome engineering

Unit 1.4. TOOLS OF BIOTECHNOLOGY: TERMS DEFINED

There are four main steps in making a transgenic organism, the process being termed **TRANSFORMATION**. There are several sources (donors) of this basic unit for

genetic manipulation. One can take a gene from another variety of the recipient, from another species, genus or family or even from another kingdom (animal, plant, fungi or bacteria). As the nucleic acid of the gene is likely to be known, one can even synthesize a gene *de novo* (afresh) in the laboratory. An important tool in the molecular biology "tool kit" for isolating genes and for assembling the elements that are introduced into recipient organisms are **RESTRICTION ENDONUCLEASES** also known as restriction enzymes. These are enzymes that cleave the double-strand of DNA at specific sites thus enabling the discrete isolation of a specific DNA sequence. These specific pieces of DNA can be joined to other pieces in a directed manner using the enzyme DNA LIGASE.

The DNA that is introduced into plant cells during the process of transformation is termed a **CONSTRUCT**. A construct contains the DNA sequence conferring the trait, the DNA elements involved in expressing the gene (the promoter, terminator and any control sequences), and one or more **SELECTION MARKER** genes. The DNA sequence conferring the trait can be either the gene of interest or DNA that transcribes to give RNA that is complementary to a sequence in the target organism; the latter is termed **antisense RNA** or **RNAi** and can induce a process called **RNA silencing.** The selection markers are used to select the transformed cells from those that are not transformed as the process of transformation can be inefficient (see below for more on selection markers). These DNA elements are assembled into one or more DNA molecules. In some transformation strategies, the introduced construct comprises both the gene and the marker(s) on the same DNA molecule; in other transformation strategies (co-transformation), the gene and markers are on separate molecules.

Having assembled the construct, one has to amplify it to provide enough material to introduce into the plant cells. This is usually performed in bacteria by introducing the construct into a bacterial **PLASMID**; this is termed **CLONING**. Various selection markers are used to select the transformed bacteria from the non-transformed bacteria. These include antibiotic resistance genes, which are of special significance in risk assessment. There are approaches that do not involve antibiotic resistance markers, such as the blue-white colour selection method and herbicide-tolerant markers. Having isolated the gene, and having made and amplified the construct, it can be introduced into recipient's cells.

The basic strategy for transforming a plant involves delivery of the construct(s) containing the gene(s) to the target material, selection of the transformed cells, and then regeneration of the transformed plant lines. As noted above, the construct comprises an expression unit containing a selectable marker (which may be different from that used in the bacterium during the amplification stage) and an expression unit containing the gene of interest.

There are three basic gene delivery systems but only two are in general use for plant transformation. The most frequently used direct transfer method is **BIOLISTICS**, also known as the gene gun, where the construct DNA is coated onto small gold or tungsten particles which are then "shot" into the target cell material. The most commonly used indirect transfer method involves **AGROBACTERIUM** (Agrobacterium-mediated transformation).

Agrobacterium is a plant-pathogenic bacterium that contains a plasmid that has the ability to insert part of its DNA into the chromosome of plants. For transformation of animals such as fish the construct is usually microinjected into fertilized eggs.

The constructs are then delivered into the target materials. Details of target materials differ between plant species and even between varieties of a species. One of the most important constraints is the ability to regenerate new plants from the target material, i.e. that the transformed cells have **totipotency.** Thus there is a wide variety of target materials, the most common being immature embryos and embryonic cell suspension (note that GUS = B-glucuronidase and GFP = green fluorescent protein, both being colour marker genes). Other targets include meristems, protoplasts and even flowers. As noted above eggs are frequently used recipient targets for animal systems. **Totipotent:** blastomeres that can develop into complete individuals when separated, or, cells capable of forming any cell type. **Blastomere** is any one of the cells formed by the first divisions of a fertilized egg. **Clone:** a group of genetically identical individuals or cells derived from a single cell by repeated asexual divisions. Or, to produce a set of identical individual cells or DNA molecules from a single starting cell or molecule.

In most of the targets the construct is inserted into nuclear DNA and hence is passed between plants during fertilization. This causes the potential risk of transgenes

spreading from transformed to non-transformed plants or animals. The chloroplasts of plants are usually inherited maternally and hence a transgene would not spread in pollen. Methods of **chloroplast transformation** are being developed which should mitigate some of the potential problems of gene flow. This approach cannot be used with animals which do not have chloroplasts. The development of mitochondrial transformation is in the very early stages both for plants and animals.

When the gene construct(s) have been delivered to the target, those cells into which there is actual integration of the input DNA have to be selected away from those that have not been transformed. There is a range of selectable markers, which, as with the selection markers for the bacteria in which the constructs are amplified, are an important aspect of biosafety risk analysis

The final stages in the production of transgenic organisms are the regeneration of the transformed material. For plants this can take a long time and requires much effort for animals, the transformed eggs are replaced into a suitable female or are cultivated in the laboratory. Then the transformed organisms have to be analysed; this will be discussed extensively during this course.

Each independently transformed individual is termed a **TRANSGENIC EVENT** and the individuals derived from a transgenic event are termed a **TRANSGENIC LINE**.

Further Reading

Ignacimuthu, S.J. 1995. Basic Biotechnology. Tata McGraw-Hill Publishing Company Limited, New Delhi. Second Reprint, 1996. 317pp.

Dictionary of Biological Terms.

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MODULE 2: MICROPROPAGATION

Unit 2.1: INTRODUCTION

Micropropagation is the practice of rapidly multiplying stock plant material to produce a large number of progeny plants, using modern plant tissue culture methods. Micropropagation is used to multiply novel plants, such as those that have been genetically modified or bred through conventional plant breeding methods. It is also used to provide a sufficient number of plantlets for planting from a stock plant which does not produce seeds, or does not respond well to vegetative reproduction.

Micropropagation is the aseptic culture of cells, pieces of tissue, or organs. It is possible to regenerate new plants from small pieces of plant tissue because each cell of a given plant has the same genetic makeup and is totipotent, that is, capable of developing along a "programmed" pathway leading to the formation of an entire plant that is identical to the plant from which it was derived. In addition to its biotechnological applications, micropropagation is used commercially to asexually propagate plants. Using micropropagation, millions of new plants can be derived from a single plant. This rapid multiplication allows breeders and growers to introduce new cultivars much earlier than they could by using conventional propagation techniques, such as cuttings. Micropropagation also can be used to establish and maintain virus-free plant stock. This is done by culturing the plant's apical meristem, which typically is not virus-infected, even though the remainder of the plant may be. Once new plants are developed from the apical meristem, they can be maintained and sold as virus-free plants.

Unit 2.2: THE PROCESS OF MICROPROPAGATION

Micro propagation differs from all other conventional propagation methods in that aseptic conditions are essential to achieve success. The process of micro propagation can be divided into four stages:

1. Initiation stage. A piece of plant tissue (called an explant) is (a) cut from the plant, (b) disinfested (removal of surface contaminants), and (c) placed on a medium. A medium typically contains mineral salts, sucrose, and a solidifying agent such as agar. The objective of this stage is to achieve an aseptic culture. An aseptic culture is one without contaminating bacteria or fungi.

2. Multiplication stage. A growing explant can be induced to produce vegetative shoots by including a cytokinin in the medium. A cytokinin is a plant growth regulator that promotes shoot formation from growing plant cells.
3. Rooting or preplant stage. Growing shoots can be induced to produce adventitious roots by including an auxin in the medium. Auxins are plant growth regulators that promote root formation. For easily rooted plants, an auxin is usually not necessary and many commercial labs will skip this step.
4. Acclimatization. A growing, rooted shoot can be removed from tissue culture and placed in soil. When this is done, the humidity must be gradually reduced over time because tissue-cultured plants are extremely susceptible to wilting.

Unit 2.2.1 Establishment

In vitro culture of plants in a controlled, sterile environment

Micro propagation begins with the selection of plant material to be propagated. Clean stock materials that are free of viruses and fungi are important in the production of the healthiest plants. Once the plant material is chosen for culture, the collection of explant(s) begins and is dependent on the type of tissue to be used; including stem tips, anthers, petals, pollen and others plant tissues. The explant material is then surface sterilized, usually in multiple courses of bleach and alcohol washes and finally rinsed in sterilized water. This small portion of plant tissue, sometimes only a single cell, is placed on a growth medium, typically containing sucrose as an energy source and one or more plant growth regulators (plant hormones). Usually the medium is thickened with agar to create a gel which supports the explant during growth. Some plants are easily grown on simple media but others require more complicated media for successful growth; the plant tissue grows and differentiates into new tissues depending on the medium. For example, media containing cytokinins are used to create branched shoots from plant buds. and it happens in a vegetative form

Unit 2.2.2: Multiplication

Multiplication is the taking of tissue samples produced during the first stage and increasing their number. Following the successful introduction and growth of plant

tissue, the establishment stage is followed by multiplication. Through repeated cycles of this process, a single explant sample may be increased from one to hundreds or thousands of plants. Depending on the type of tissue grown, multiplication can involve different methods and media. If the plant material grown is callus tissue, it can be placed in a blender and cut into smaller pieces and re -cultured on the same type of culture medium to grow more callus tissue. If the tissue is grown as small plants called plantlets, hormones are often added that cause the plantlets to produce many small offshoots that can be removed and re -cultured, these are all 5 types

Unit 2.2.3: Pre-transplant

Banana plantlets transferred to soil (with vermi-compost) from plant media. This process is done for acclimatization of plantlets to the soil as they were previously grown in plant media. After growing for some days the plantlets are transferred to the field.

This stage involves treating the plantlets/shoots produced to encourage root growth and "hardening." It is performed *in vitro*, or in a sterile "test tube" environment.

"Hardening" refers to the preparation of the plants for a natural growth environment. Until this stage, the plantlets have been grown in "ideal" conditions, designed to encourage rapid growth. Due to lack of necessity, the plants are likely to be highly susceptible to disease and often do not have fully functional dermal coverings and will be inefficient in their use of water and energy. In vitro conditions are high in humidity and plants grown under these conditions do not form a working cuticle and stomata that keep the plant from drying out, when taken out of culture the plantlets need time to adjust to more natural environmental conditions. Hardening typically involves slowly weaning the plantlets from a high-humidity, low light, warm environment to what would be considered a normal growth environment for the species in question. This is done by moving the plants to a location high in humidity.

Unit 2.2.4: Transfer from culture

In the final stage of plant micro propagation, the plantlets are removed from the plant media and transferred to soil or (more commonly) potting compost for continued growth by conventional methods.

This stage is often combined with the "pre transplant" stage.

UNIT 2.3: ADVANTAGES OF MICROPROPAGATION

Micropropagation has a number of advantages over traditional plant propagation techniques:

- The main advantage of micro propagation is the production of many plants that are clones of each other.
- Micro propagation can be used to produce disease-free plants.
- Micro propagation produces rooted plantlets ready for growth, saving time for the grower when seeds or cuttings are slow to establish or grow.
- It can have an extraordinarily high fecundity rate, producing thousands of propagules while conventional techniques might only produce a fraction of this a number.
- It is the only viable method of regenerating genetically modified cells or cells after protoplast fusion.
- It is useful in multiplying plants which produce seeds in uneconomical amounts, or when plants are sterile and do not produce viable seeds or when seed can't be stored (vgr. recalcitrant seeds).
- Micro propagation often produces more robust plants, leading to accelerated growth compared to similar plants produced by conventional methods - like seeds or cuttings.
- Some plants with very small seeds, including most orchids, are most reliably grown from seed in sterile culture.
- A greater number of plants can be produced per square meter and the propagules can be stored longer and in a smaller area.

UNIT 2.4: DISADVANTAGES OF MICROPROPAGATION

Micro propagation is not always the perfect means of multiplying plants. Conditions that limit its use include:

- It is *very* expensive, and can have a labour cost of more than 70%

- A monoculture is produced after micro propagation, leading to a lack of overall disease resilience, as all progeny plants may be vulnerable to the same infections.
- An infected plant sample can produce infected progeny. This is uncommon if the stock plants are carefully screened and vetted to prevent culturing plants infected with virus or fungus.
- Not all plants can be successfully tissue cultured, often because the proper medium for growth is not known or the plants produce secondary metabolic chemicals that stunt or kill the explant.
- Sometimes plants or cultivars do not come true to type after being tissue cultured. This is often dependent on the type of explant material utilized during the initiation phase or the result of the age of the cell or propagule line.
- Some plants are very difficult to disinfest of fungal organisms. The major limitation in the use of micro propagation for many plants is the cost of production; for many plants the use of seeds, which are normally disease free and produced in good numbers, readily produce plants (see orthodox seed) in good numbers at a lower cost. For this reason, many plant breeders do not utilize micropropagation because the cost is prohibitive. Other breeders use it to produce stock plants t hat are then used for seed multiplication.

Mechanisation of the process could reduce labour costs, but has proven difficult to achieve, despite active attempts to develop technological solutions.

REFERENCES

1. "Micropropagation - Definitions from Dictionary.com". dictionary.reference.com. Retrieved 2008-03-17.
2. Nishibayashi Nature Chemistry Volume: 3, Pages: 120–125 Year published:(2011doi:10.1038/nchem.906

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MODULE 3: MUTATION

Adapted from http://en.wikipedia.org/wiki/Mutation

In molecular biology and genetics, **mutations** are changes in a genomic sequence: the DNA sequence of a cell's genome or the DNA or RNA sequence of a virus. They can be defined as sudden and spontaneous changes in the cell. Mutations are caused by radiation, viruses, transposons and mutagenic chemicals, as well as errors that occur during meiosis or DNA replication. They can also be induced by the organism itself, by cellular processes such as hypermutation.

Mutation can result in several different types of change in sequences; these can either have no effect, alter the product of a gene, or prevent the gene from functioning properly or completely. Studies in the fly *Drosophila melanogaster* suggest that if a mutation changes a protein produced by a gene, this will probably be harmful, with about 70 percent of these mutations having damaging effects, and the remainder being either neutral or weakly beneficial.[4] Due to the damaging effects that mutations can have on genes, organisms have mechanisms such as DNA repair to prevent mutations.

Mutations can involve large sections of DNA becoming duplicated, usually through genetic recombination. These duplications are a major source of raw material for evolving new genes, with tens to hundreds of genes duplicated in animal genomes every million years. Most genes belong to larger families of genes of shared ancestry.[7] Novel genes are produced by several methods, commonly through the duplication and mutation of an ancestral gene, or by recombining parts of different genes to form new combinations with new functions.

Changes in chromosome number may involve even larger mutations, where segments of the DNA within chromosomes break and then rearrange. In evolution, the most important role of such chromosomal rearrangements may be to accelerate the divergence of a population into new species by making populations less likely to interbreed, and thereby preserving genetic differences between these populations.

Nonlethal mutations accumulate within the gene pool and increase the amount of genetic variation.[20] The abundance of some genetic changes within the gene pool can be reduced by natural selection, while other "more favorable" mutations may accumulate and result in adaptive changes.

For example, a butterfly may produce offspring with new mutations. The majority of these mutations will have no effect; but one might change the color of one of the butterfly's offspring, making it harder (or easier) for predators to see. If this color change is advantageous, the chance of this butterfly surviving and producing its own offspring are a little better, and over time the number of butterflies with this mutation may form a larger percentage of the population.

Neutral mutations are defined as mutations whose effects do not influence the fitness of an individual. These can accumulate over time due to genetic drift. It is believed that the overwhelming majority of mutations have no significant effect on an organism's fitness. Also, DNA repair mechanisms are able to mend most changes before they become permanent mutations, and many organisms have mechanisms for eliminating otherwise permanently mutated somatic cells.

Unit 3.1. Causes of Mutation

Two classes of mutations are spontaneous mutations (molecular decay) and induced mutations caused by mutagens.

Unit 3.1.1 Spontaneous mutation

Spontaneous mutations on the molecular level can be caused by:[21]

- Tautomerism – A base is changed by the repositioning of a hydrogen atom, altering the hydrogen bonding pattern of that base resulting in incorrect base pairing during replication.
- Depurination – Loss of a purine base (A or G) to form an apurinic site (AP site).
- Deamination – Hydrolysis changes a normal base to an atypical base containing a keto group in place of the original amine group. Examples include C → U and A → HX (hypoxanthine), which can be corrected by DNA repair

mechanisms; and 5MeC (5-methylcytosine) → T, which is less likely to be detected as a mutation because thymine is a normal DNA base.

- Slipped strand mispairing – Denaturation of the new strand from the template during replication, followed by renaturation in a different spot ("slipping"). This can lead to insertions or deletions.

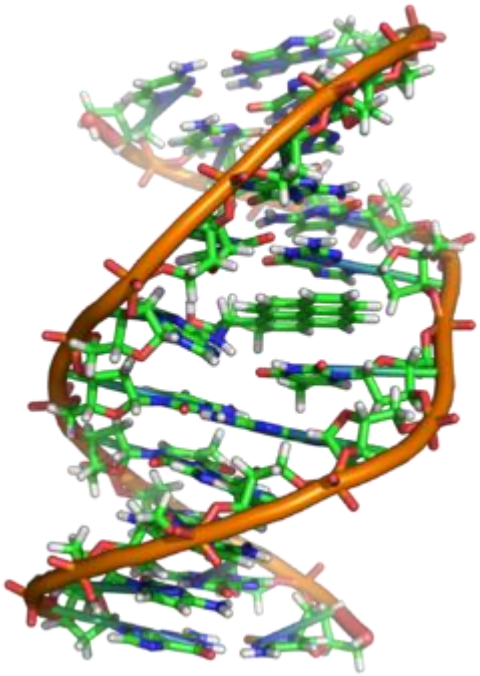

Figure: A covalent adduct between benzo[*a*]pyrene, the major mutagen in tobacco smoke, and DNA

Unit 3.1.2. Induced mutation

Induced mutations on the molecular level can be caused by:

- Chemicals
  - Hydroxylamine NH_2OH
  - Base analogs (e.g. BrdU)
  - Alkylating agents (e.g. *N*-ethyl-*N*-nitrosourea) These agents can mutate both replicating and non-replicating DNA. In contrast, a base analog can only mutate the DNA when the analog is incorporated in replicating the DNA.

Each of these classes of chemical mutagens has certain effects that then lead to transitions, transversions, or deletions.

- Agents that form DNA adducts (e.g. ochratoxin A metabolites)
- DNA intercalating agents (e.g. ethidium bromide)
- DNA crosslinkers
- Oxidative damage
- Nitrous acid converts amine groups on A and C to diazo groups, altering their hydrogen bonding patterns which leads to incorrect base pairing during replication.

- Radiation
  - Ultraviolet radiation (nonionizing radiation). Two nucleotide bases in DNA – cytosine and thymine – are most vulnerable to radiation that can change their properties. UV light can induce adjacent pyrimidine bases in a DNA strand to become covalently joined as a pyrimidine dimer. UV radiation, particularly longer-wave UVA, can also cause oxidative damage to DNA. Mutation rates also vary across species. Evolutionary biologists have theorized that higher mutation rates are beneficial in some situations, because they allow organisms to evolve and therefore adapt more quickly to their environments. For example, repeated exposure of bacteria to antibiotics, and selection of resistant mutants, can result in the selection of bacteria that have a much higher mutation rate than the original population (mutator strains).

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MODULE 4:

CONSTRAINT ON CROP PRODUCTION AND THE CIRCUMVENTING IMPACT OF BIOTECHNOLOGY

Unit 4.1: CONSTRAINTS ON CROP PRODUCTION

You may ask the question, "Why are transgenics needed?"

Transgenic crops are satisfying needs which crop varieties produced by conventional breeding methods cannot. Some people already affirm that genetic engineering appears as an indispensable tool for crop management and disease control systems, and for the increase by seventy-five percent of agricultural production from now to 2020 in order to be able to tackle the high increasing rate of the African population, objectives that classical breeding methods, alone, cannot attain.

Agricultural productivity is severely limited by a large number of constraints that are **Biotic** (arthropods, nematodes, diseases, weeds, rodents, birds); **Abiotic** (drought, soil fertility, mineral toxicity); and **Infrastructural** (low price to farmers and inability to compete with subsidized imports, high price of pesticide and fertilizer inputs, poor transportation and storage systems).

The issue of food and feed quality, which can be slowly and subtly destructive, situation that is only recognized by medical epidemiologists and by measurements in material stored on the farm. Some fungal infections hardly reduce yields but produce mycotoxins. Except for export crops, these have largely been ignored; the low chronic levels of toxins in farmers' diets have not been addressed. Expensive fungicides could be used for export crops, but the lack of recognition of the problem, together with the prohibitive expense of fungicides, has allowed the mycotoxin problems to increase greatly. Chronic low levels of mycotoxins in food and feed lead to poor utilization of food – i.e. malnourishment of people and slow growth rate in livestock and poultry. Higher rates lead to various cancers and death.

Unit 4.1.1: Biotic constraints where conventional technologies have been inadequate

Crops have been heavily selected by farmers and bred by scientists to overcome indigenous diseases and insects not found elsewhere. They have selected for ability to compete with weeds – with a considerable loss of yield until the green revolution rectified this, thanks to the availability of cheap cost-effective herbicides. Indeed,

pesticides are the major way of dealing with the biotic stresses, where they work. Still the preferred methods of dealing with these constraints are in seed technologies, where the crop can deal with the pest directly, through genetic means. The only caveat to this is there must not be a yield penalty that is greater than the cost of pesticides.

Unit 4.1.2: Disease constraints

Fungal and viral pathogens of plants are continually evolving and plant breeders are always having to play "catch up" to produce resistant varieties. There are an increasing number of cases where breeders cannot keep up, or where there is no inherent resistance within the crop, especially with some viral diseases.

Unit 4.1.3: Insect constraints

Even in the developing world insecticides are used sparingly, mainly on high value fruit and vegetable crops, as well as cotton, and less frequently on row crops such as maize. Insect pest infestations in staple row crops have either been “accepted” because insecticides are too expensive, or treated with low amounts of insecticides, often applied using methods that would be deemed unsafe and unacceptable in the developed world, i.e. in contravention of the pesticide label, e.g. a drop or pinch of insecticide applied by hand into the cup shaped whorl of leaves. There are also cases where insecticide resistance has evolved. Insects are also vectors of viral diseases. When insecticides are applied the farmer often does not use the advised application and protection advice with consequent risks to the environment and to his health.

Unit 4.1.4: Weed constraints

Weeding is predominantly a backbreaking task for women, often consuming 80% of their waking hours (Akobundo, 1991 Weeds in human affairs in sub-Saharan Africa,

Weed Tech. 5, 660-690). Weed issues have been largely ignored both on the farm and in the research community, possibly due to an element of male chauvinism. Hand weeding has created a niche for weeds that could not be hand weeded, and which could not be directly killed by herbicides, without killing the crop. Where herbicides have been used, weeds have evolved resistance or new weed species have appeared that could not be selectively controlled by herbicides. In some cases resistance to herbicides has evolved and spread over millions of hectares, e.g. resistance of *Phalaris minor* to the herbicide isoproturon in India.

Unit 4.1.5: Abiotic constraints

Overgrazing and agricultural practices such as ploughing under inappropriate conditions can lead to soil degradation and severe erosion problems.

The use of fertilizer has become widespread within the developed world, but optimum amounts are typically too expensive for farmers in the developing world. Adding fertilizer at times of high rainfall can lead to leaching into ground waters, causing severe environmental problems. Conversely, the law of conservation of matter decrees that you cannot create minerals where they are lacking. Be that as it may, some plants and microorganisms can extract mineral nutrients that are in unavailable forms, others are more efficient users of minerals, meaning that there must be genes that provide higher efficiency and mobilization of nutrients. Degraded soils are often acid or saline, have high levels of lead, aluminium or other toxic ions, limiting the species that can exist on such poor substrates. Liming and other amenities are often not an economic option. The transfer of genes from species that are tolerant of such pollutants to crop species can increase crop biodiversity – i.e. the options of the farmer.

Drought stress

Transient drought, when the duration between rains is all to long, is a common occurrence, with consequential crop losses. In dry areas without an irrigational back-up system, it can mean crop loss. This is a major constraint to dryland agriculture. As

some species can withstand this, there must be genes available. General drought, i.e. regions where the annual rainfall is very low presents a separate problem – requiring species that yield a crop (albeit low yields) or their relevant genes for movement into other crops.

Water availability for agriculture is an increasing major constraint due to climate change (global warming) and the damands of a growing population.

Cold stress

This can be either transient (early frost) or long term (very cold winters) and limits the species that can be cultivated. Thus, agriculture in areas prone to this constraint requires species that yield a crop (albeit low yields) or their relevant genes for movement into other crops.

Unit 4.1.6: Conclusion

It is necessary to ascertain whether biotechnologies can supply rapid, safe, cost effective solutions to the intractable biotic and abiotic constraints. The institutional and infrastructure constraints to agriculture are amenable to positive human intervention, and could facilitate rapid adoption of the yield and quality enhancing biotechnologies in later lectures. Whether biotechnological solutions are employed is a matter of consumer demand and need, and the resolve of politicians and regulators to deal with these issues in a science and fact based manner with due resolve.

Unit 4.2: CONTRIBUTION OF BIOTECHNOLOGY TO CROP PRODUCTION AND RELATED FIELDS

In the field of agriculture, biotechnology has contributed to crop improvement by developing salt and drought resistant crops, developing crop varieties which can fix atmospheric nitrogen, improving crop varieties for higher photosynthetic efficiency,

improving the quality and quantity of storage proteins, and production of high yielding hybrid seeds. Circumventing

Further, it has contributed to crop protection through development of disease resistant varieties of transgenic plants which offer protection against virus, bacteria, fungi, nematodes and insects, development of herbicide resistant plants and development of recombinant DNA based diagnostic reagents for early identification and treatment of viral, bacterial and fungal diseases. Tissue culture techniques have helped in the propagation of forest plants such as bamboo, teak, Eucalyptus, etc. and economically important plants such as banana, *Brassica* and even biofuel. Biotechnology has also helped in the production of single cell proteins and biofertilizers. In the field of energy, biotechnology has helped in the conversion of cellulosic and agricultural waste to produce fuels such as ethanol and butanol by cloning active enzyme systems for their efficient degradation and subsequent fermentation.

Biotechnology has helped in the production of biogas from agricultural and animal wastes by engineering methanogenic bacteria. It has also helped in the production of oil producing bacteria. Biotechnology has also played an important role in waste treatment by developing high capacity bacteria to degrade cellulose and lignin, to degrade metals and cyanide, to clean up oil, to destroy TNT (i.e. **Trinitrotoluene,** a useful explosive material with convenient handling properties), to degrade chlorinated organic wastes, etc. Thus, biotechnology has contributed to the welfare of humanity in many and varied ways. Biotechnology has become an important and integral part of any life science today.

Unit 4.3: TERMS DEFINED

Transgenic plants: plants into which genes from another species have been deliberately introduced by genetic engineering. The bacterium *Agrobacterium tumefaciens* carrying the required gene is often used to infect and transmit the gene to cultured plant tissue from which a transgenic plant can be regenerated.

Recombinant DNA: DNA produced by joining together *in vitro* genes from different sources or which has in some way been modified *in vitro* to introduce novel genetic information. Or, DNA produced as a result of natural genetic recombination.

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MODULE 5

PRODUCTION OF DISEASE-FREE AND DISEASE/PEST-RESISTANT PLANTS

UNIT 5.1: PRODUCTION OF PEST RESISTANT VARIETIES

Control of Plant Viruses

There are currently three approaches to controlling plant viruses. Pesticides are used to control the invertebrate vector. This can be environmentally damaging (see the white insecticide deposits on the rice) and many insects develop resistance to the insecticides. The second is clean agronomic practices such as removal of sources of infection. This can be effective for perennial crops but in others such as attempts to control *Cacao swollen shoot virus* did not succeed. This approach does not work for many annual crops in tropical countries where young and old crops are grown side-by-side. The third approach is the breeding of resistance into the crop; this can be either by conventional breeding or by GM. In conventional breeding the resistance can be to either the virus or the vector. However, the great variability of many viruses results in the plant breeder having to play "catch up" with new virus variants that break the resistance gene. This figure shows an example of this with the reaction of various genes bred into tomato against variants of *Tomato mosaic virus* (related to *Tobacco mosaic virus*). In practice, when one resistance gene was widely deployed a

new strain of the virus arose which broke the resistance. This has led to considerable efforts in applying GM approaches to controlling plant viruses.

In some of the earliest experiments with transgenic plants it was shown that the transgenic expression of some viral sequences could confer resistance to the cognate virus. The initial experiments on transgenic virus resistance involved the expression of the viral coat protein (termed coat-protein protection); subsequently, expression of other viral gene products, either unmodified or mutated in important regions, have given some protection. The results from some of these protein-expressing systems did not make sense as resistance was also obtained using some non-coding sequences (the controls on the experiments). Thus it appeared that the expressed nucleic acid was playing an important role. In some of these experiments, the expressed RNA was complementary (antisense) to the viral mRNA, in others it was in the same sense.

This, and other observations, led to the realization that plants (and other organisms) have a previously unrecognised defence system against "foreign" nucleic acids - termed GENE SILENCING. There are two inter-related forms of gene silencing, one that operates on the chromosomal DNA (TRANSCRIPTIONAL GENE SILENCING) and the other on the RNA in the cytoplasm (POST-TRANSCRIPTIONAL GENE SILENCING or PTGS). PTGS, also known as RNA silencing, quelling (in fungi) and RNAi (RNA interference) (in animals), has proven to be a very important feature in transgene technology. At some stage in the replication of the "foreign" RNA, double-stranded RNA (dsRNA) is formed and the plant (or animal) recognizes this as being foreign. The right-hand part of the figure shows what happens to this dsRNA. It is targetted by an enzyme called DICER which cuts it into pieces of 21 to 25 nucleotides (termed small interfering RNAs or SiRNAs). These then combine with various proteins to form to form a macromolecules termed the RNA-induced silencing complex (RISC). Proteins in the RISC unwind the double-stranded SiRNAs which then give single-standed SiRNAs of both polarities. The SiRNA then anneals to the target RNA (which has the same sequence as that from which it originated) making further dsRNA which is in turn targetted by DICER. Thus the "foreign" single-stranded RNA is degraded.

The importance of ds RNA in the RNA silencing process has led to constructs that transcribe to give this molecule. In this construct the coding sequence is duplicated to

give an inverted repeat, the two parts being separated by a non-coding region (intron). This approach is opening up a whole new transgenic strategy not only against viruses but also using RNA silencing to "switch off" the expression of undesirable genes, e.g. for allergenic gene products.

There are several examples of transgenic protection against viruses in the field. These two examples were intended to involve coat protein protection but in the light of finding on RNA silencing, there may be uncertainty as to how they operate. However, as pointed out below, it is important in making a biosafety determination to know which mechanism is involved.

Several main areas of concern have been raised about the deployment of plants transgenic in virus sequences. The main ones are: Interactions with other viruses; Suppression of gene silencing; Spread of transgenes to other plants; Possible effects on humans and other animals; Stability of the transgene.

Although the transgene viral sequence will give protection against the donor virus (and possibly closely-related viruses) it will not do so against unrelated superinfecting viruses. Thus there is potential for at least three types of interaction between the transgene viral sequence and the superinfecting virus:

- Firstly, as the coat protein of a virus can determine the specific interactions with the vector (insect or nematode) the concern is that the transgenically-expressed coat protein can encapsidate (termed heteroencapsidate) the genome of the superinfecting virus thus changing the transmission properties of that virus. This happens occasionally with natural double infections of non-transgenic plants but there is no evidence that it is more prevalent with transgenic plants.
- Secondly, some combinations of viruses cause more severe symptoms than the sum of those of the two original viruses - termed synergy. The concern is that the transgene will interact with the superinfecting virus exacerbating the symptoms. Both these concerns relate to transgenes that produce proteins. Thus the use of the RNA silencing approach is likely to be much more efficient at protecting plants against virus infection and so these concerns should not be important in the future.

- The third and main concern about the use of viral transgenes that produce just nucleic acid is that there could be recombination between the transgene RNA and that of the superinfecting virus giving rise to a new "supervirus". This would happen during replication of the viral nucleic acid, usually by the replicase enzyme switching strands. It would only potentially cause a problem if the recombinant virus was viable and had a selective advantage over the superinfecting virus. There are several examples of recombination between two viruses in non-transgenic situations resulting in new viruses; one case is the devastating Uganda variant of African cassava mosaic virus that devastated the cassava crop in East Africa in the 1990s.

There are further concerns of potential hazards relating to plant viruses. The gene silencing (RNA silencing or RNA interference) phenomenon is raising two concerns about viruses and transgenic plants. Firstly, as mentioned above, the CaMV 35S promoter is widely used and infection of oilseed rape with CaMV has been shown to silence the expression of herbicide resistance expressed from that promoter. The second concern relates to a property of successful viruses. As RNA silencing is a defence mechanism against "foreign" nucleic acids successful viruses have to suppress it to be able to overcome this host defense. Various viral genes have been identified as suppressors of RNA silencing. The question is does this suppression of gene silencing occur in an infection of a transgenic plant switch off a silencing construct?

Further concerns about transgenic protection against viruses are more general and include possibility of spread of the transgene to other plants causing effects to the environment (virus resistant plants in the ecosystem), possible effects of the expression of viral sequences on humans and other animals (e.g. allergies) and stability of such transgenes. These will be discussed in other sections of the course.

As much is known about the detailed molecular biology it should be possible to develop systems to minimize or obviate any hazard. Thus, one can make viral constructs that do not contain a potentially hazardous sequence, for instance the amino acid sequences that control insect vector specificity or nucleic acid sequences that are involved in recombination. This is termed "sanitising the construct".

However, most of these hazards are hypothetical and the best approach is to monitor releases, a subject that will also be discussed later.

UNIT 5.2: FUNGAL PATHOGENS

There is a wide range of fungal diseases that can affect all parts of plants and. Some fungi cause leaf damage either leading to a quick death of the infected leaf as in potato blight or slower death but significant effects on the plant as in mildews and rusts. Fungi such as Botrytis cause rots in storage organs and fruits. Scab fungus damages potatoes with loss of quality and storage ability; similarly, mildews can cause loss of quality in fruits such as grapes. Cereals can be affected by smuts and ergot; ergot is very important as it contains various alkaloids, ingestion of which can cause constriction of blood vessels, abortions, hallucinations and mental aberrations. Fungi can also affect and kill trees. One of the most notorious fungi is Potato late blight, which caused the Irish Potato famine leading to a mass migration primarily to the USA. Fungal infections can also produce mycotoxins.

Unit 5.2.1. Conventional Control of Fungal Pathogens

There are currently three approaches to controlling fungal diseases. The most widespread is the application of fungicides of which there are three types: sterilants and fumigants which are usually used to sterilize soil or fumigate enclosed spaces; protectants which are sprayed to prevent infections; therepeutics which are used to "cure" infections. There has been a continuous increase in the use of fungicides over the last 20 years or more (in the USA rising from 19.5 million kg in 1983 to 35.5 million kg in 1993) and the use of this input is increasing worldwide. There are several problems arising from the continuous use of fungicides: resistant variants of pathogenic fungi are selected, there is an unquantified ecological impact; there are food safety concerns and there are potential health hazards for the farmer.

The term "pest" was originally applied to animals (insects, nematodes, mammals and birds) that caused crop losses; the term "disease" applied to viruses, bacteria and fungi. However, the term "pesticide" is now generally used for chemicals that cause biotic losses in crops.

The second approach to controlling fungal diseases, as with virus diseases, is good agronomic practice such as removing sources of infection or growing the crop either in places where the pathogen does not occur or at a time of the year when it is not spreading. The details of this approach vary with pathogen, agronomic system, climate and other conditions.

Thirdly, conventional breeding for resistance to specific fungal diseases has been used for many years. There are three major genetic traits that are used in breeding programmes:

1. Resistance can be given by various characters of plant structure such as a thick and waxy cuticle (outer skin of the plant leaves and stem) or a leaf structure that does not accumulate water drops (the spores of many fungi require water for germination).
2. When a fungus infects a plant there is chemical communication between the two, the fungus determining a suitable host and the plant trying to resist fungal infection. Some resistance genes enhance the plant defence.
3. Although not much is known about most of such plant defences one has been studied in detail. This is the hypersensitive response in which the plant responds by killing off its cells around the initial stages of infection. This response is due to the interaction of a plant resistance R gene and the fungal elicitor gene. The understanding of this interaction has led to the gene-for-gene theory, one fungal (elicitor) gene product interacting with one host (resistance) gene product. Many plant R genes (which can also give resistance to bacteria and viruses) have been characterized and shown to have various basic features in common such as nucleic acid binding domain (NB) and leucine-rich repeat (LRR). One of the problems with the gene-for-gene situation is that that a small change in the fungal elicitor gene can overcome the plant R gene and thus resistance breaks down. The breeder is having to find new sources of R genes for variants of fungal pathogens - the actual

deployment of an R gene into the field can often select resistance-breaking variants. The problem then comes of finding sources of R genes in species that can be successfully crossed with the crop of interest.

Unit 5.2.2. GM Control of Fungal Pathogens

As with viruses, an understanding of the interactions between the pathogen and host has led to developing transgenic approaches to protecting crop plants against fungal infections. However, the interactions are more complex than those of viruses and to date (2006) there have been no commercial releases of transgenic fungus-resistant plants. There are several approaches being used in laboratory studies on conferring protection against fungi with transgenes.

1. R genes can be isolated from resistant varieties of the same species or from closely related species and put into the desired crop. An example of this is the resistance gene to potato late blight isolated from a related species and inserted into potato. The colinearity or synteny of genes in chromosomes of related species (discussed in Unit 1.3) makes the identification and isolation of such genes easier. This approach of the transgenic movement of R genes between plants has several advantages such as shortening breeding programmes, bypassing sexual incompatability barriers and being able to "stack" R genes (i.e introduce several R genes into an elite variety).
2. Because R genes have a common structure and their specificity is due to minor variation in that structure, another source of resistance genes is to modify the R gene in the laboratory.
3. The interactions between the fungal elicitor and the R gene lead to a cascade of metabolic reactions resulting in hypersensitivity and other host responses. Modification of this pathway is showing some potential for conferring resistance. Similarly, the enhancement of other plant responses to pathogen infection is showing promise.
4. Many plants produce small peptides that have anti-microbial properties. Several of these have been isolated, characterised, their genes identified and

inserted into the crop species. Similarly, anti-microbial peptide genes from dahlia and onion have been put into bananas giving protection against the devastating black Sigatoka fungus.

5. Various other genes are being tested for protecting transgenic plants against fungal infection. The cell walls of fungi are made of chitin and glucans and one of the most promising approaches is to attack the fungal cell wall with chitinases and gluconases.

UNIT 5.3: BACTERIAL PATHOGENS

Bacterial pathogens are not very important in temperate countries but can be very important in tropical countries. Plant pathogenic bacteria cause a variety of symptoms usually wilts and rots; one, Agrobacterium, causes crown gall due to transfer of part of a plasmid DNA to the host. The current control measures are similar to those of fungal pathogens comprising agronomic approaches such as using clean planting material and planting at times or places to avoid the disease together with breeding for resistance. Several R genes are proving important in breeding programmes. Some studies are being made on contolling bacterial pathogens by Genetic modification of crop plants using approaches similar to those described above for fungal pathogens. One recent success has been the transfer of the rice bacterial blight R gene, Xa21, from a basic rice variety into elite cultivars where it gave broad spectrum resistance to strains of the pathogen. Some Xa21 transgenic lines should be released in China during 2006 or 2007.

Unit 5.4. BIOSAFETY CONSIDERATIONS

The main biosafety concerns about crop plants transgenically protected against fungi and bacteria are on the food and health safety of novel gene products (e.g. chitinases) and spread of resistance genes into the environment. These will be discussed in Units 4.1 and Unit 5.1.

UNIT 5.5 INSECT AND NEMATODE RESISTANCE

Unit 5.5.1: INTRODUCTION TO PEST INSECTS

As well as losses to crops caused by diseases, animals such as insects and nematodes are significant biotic factors. There are two basic types of feeding behaviour by insect pests. Some pests, such as aphids, leafhoppers and whitefly obtain their food by piercing plant cells with their mouthparts and the sucking out the cells contents. Such insects often live in large colonies and cause damage not only by killing plant cells but also as major vectors of plant viruses. The other type of insect feeding behavour is chewing plant leaves, stems or other parts of the plant. Most of these insects are beetles (Coleoptera), flies (Diptera) or Moths (Lepidoptera) and, in most cases, it is the larvae that do the damage. Insects feed on seeds or on the inside of the plant stem; the feeding damage on seeds can lead to fungal infection and thus to mycotoxin production. The larvae of insects feed on the inside of cotton fruiting structures (cotton bolls from which cotton is extracted) causing considerable economic losses; losses are more considerable in developing countries where less insecticides are used.

There are four basic approaches to controlling pest insects. The most widespread method is the application of insecticides, which causes many problems. As well as the potential hazard to human health, most insecticides are not completely specific to the target pest, also killing non-target beneficial insects; they can enter the food chain causing hazards to birds and mammals. Furthermore, it is difficult to control pests that feed inside plants, such as stem borers, with insecticides. The continual use of insecticides can lead to the target insects developing resistance to the chemical.

As with plant pathogens, farmers adopt a range of cultural techniques to try to control insect predation. Attempts to avoid insects are made by planting their crops at times or in places where insect populations are minimal. Mixed cropping can reduce insect populations; for instance some Andean farmers grow maize and lupins together, which reduces insect damage on the corn.

Mixed cropping leads onto another approach to pest insect control, that of integrated pest management (IPM). There have been many IPM schemes but relatively few can be regarded as being entirely successful. This is because of the complexity of the

ecosystems that the schemes are attempting to influence and the fact that there are many varying external factors continuously affecting the ecosystems.

Fourthly, as with controlling pathogens, plant breeding has been extensively used in attempts to introduce characters conferring resistance to pest insects into crop plants. Since plants can not run away from danger that have developed three types of defence against insects and other herbivores. Plant secondary metabolites have been adapted to either poison or deter pests to give effectively internal chemical control. However, as many of these may cause damage to humans they have to be reduced or eliminated over the years in breeding programmes. The structure of plants can provide physical barriers that inhibit pest insects and plants can grow in such a way as to compensate for damage caused by pests. Examples of this are hairy leaves or leaves with glands that exude a stick liquid.

Unit 5.5.2: GM CONTROL OF PEST INSECTS

One of the two transgenic traits that have been commercially deployed in a widespread manner is that conferring protection against certain insect pests. There are two main modes of operation of these transgenes. The most frequently used is that which inhibits the insects' digestion processes either by damaging the gut wall or by inhibiting digestion, thereby starving it. There are two genes used to damage insect pest gut walls, the most widespread being the toxin encoded by a bacterium named *Bacillus thuringiensis*, termed Bt toxin.

The basic details of Bt and its endotoxin are shown. There are different forms of the Bt toxin protein, each with a different range of target insects. It is important to note that most of these target insects are those that chew plants and usually not those that feed by piercing and sucking. Another important point is that each toxin is limited in its effective range and is not toxic to most non-target organisms. Recently, studies have shown that it is possible to modify the Bt toxin molecule to change its target and tailor it to specific requirements. The transgenic expression of the Bt gene means that the toxin is produced throughout the plant and is very effective against pests that live within the stem or in cotton bolls; thus transgenic expression of Bt gives good levels

of pest control in commercially released varieties. It is now being considered for controlling pests in food crops such as rice.

The widespread deployment of Bt toxin-expressing crops has revealed various advantages and also raised a number of concerns. Among the advantages are greatly increased yields, reduced insecticide input and a reduction in the production of mycotoxins. The concerns fall under four headings:

1. Possible effects to the health of humans and other animals have been studied in depth and, in spite of various reports there is no firm evidence for any bad effect.
2. There have been concerns about gene flow to the environment - these will be considered later.
3. Similarly there have been concerns about effects on non-target species exemplified in the Monarch Butterfly episode. Once again there is no direct evidence for such effects, especially when one measures them against a baseline of the conventional agronomic practice of insecticide spraying. See also abstract under Additional Reference 1 below.
4. There are worries about target insects developing resistance against Bt toxin and there is some recent evidence that this may be happening in limited cases. Ways of mitigating this will also be discussed in Module 5.

The second gene used to affect the insect's digestive system is an agglutinin from the snowdrop (*Galanthus nivalis*) (GNA) which targets sap-sucking insects. Although the transgenic expression of GNA shows considerable promise in controlling several important plant pests there have been no commercial releases on it yet. Concerns have been expressed as to potential hazards to human health and its use has been controversial: for the so-called Pusztai GNA-potato controversy. In view of the "bad press" concerning GNA, the possibilities of using other plant lectins is being explored.

As well as affecting digestion by damaging or blocking the insect gut cell wall, the actual process of digestion can be inhibited. Insects use various enzymes to digest their food in their gut such as proteases that digest proteins. There are various forms of proteases, the most important ones in insects being trypsins, chymotrypsin, alpha-

amylase and cysteine; inhibitors for each of them are known. Experiments are under way to express these protease inhibitors in plants so that insects feeding on them cannot digest their food. The concerns about protease inhibitors are similar to those on Bt toxin and especially on possible effects on non-target species.

Various other approaches to protecting crop plants against pest insects are being explored. These include the isopentenyl transferase gene from bacteria which affects insect cytokynin biosynthesis, toxins from spiders, scorpions and wasps and proteins from insect endoparasites. All these are at the experimental stage and after assessing their effectiveness, large-scale safety checks have to be undertaken. Also they will have to be acceptable to the public and some of the toxins mentioned above may prove difficult to "sell". However, it is possible that some of these genes or yet others may reach the stage of commercial release reasonably soon.

Unit 5.5.3: INTRODUCTION TO NEMATODES

Nematodes are small worm-like organisms (eel-worms), some species of which feed on plants. While some nematode species feed on the aerial parts of plants most are underground attacking the roots; some are vectors of viruses. As most of the damage by nematodes is underground relatively little is known about them, although crop losses due to them are probably very high; there is an estimate that they may cause losses of more than $1 billion per year to world agriculture. They are very difficult to control. Chemical nematicides are highly toxic and difficult to apply and are only economic in special situations such as small areas of high value crops. The cultural techniques usually involve avoiding nematode-infested soil. There are some sources of natural resistance and some R genes such as were described for fungi and bacteria.

Unit 5.5.5: GM CONTROL OF NEMATODES

Experiments with transgenic approaches to resistance using the digestion inhibitor cystatin are showing promise. However, as with the digestion inhibitor approach for insects, much work on biosafety aspects needs to be undertaken.

Unit 5.5.6: HERBICIDE TOLERANCE

Herbicide resistance is the ability of a plant to survive and reproduce following exposure to a dose of herbicide that would normally be lethal to the wild type. Resistance may occur naturally due to random and infrequent mutations, or may be induced through conventional or recombinant technologies (Prather *et al*., 2000).

In natural populations, herbicide-resistant plants are rare. Resistant plants likely will persist in infested fields for many years, even in the absence of any additional selection with the herbicide. There is no evidence that herbicides cause the genetic mutations that lead to herbicide resistance.

Producing plants that are tolerant to specific herbicides is actually one of the largest uses of plant genetic engineering. Herbicide tolerant crops "will allow non-persistent herbicides (e.g. glyphosphate) to be more widely used and will permit postemergence spraying of herbicide-resistant crops." (Snow *et. al*, 1997). It is also pointed out that genetically engineered crops reduced the use of pesticides and are thus environmentally beneficial (Wauchope *et al*., 2002; Hin *et al*., 2001; Fernandez-Cornejo *et al*., 2001). The first decline in pesticide sales was observed in 1999 (James, 2001). The major factor in this decline is the increased area of transgenic crops: Round Up Ready and Liberty Link transgenic soybean, cotton, maize and oilseed rape (James, 2001).

Table 1 Some of the herbicide resistant crops under development, grouped according to the techniques of development (Connor and Field, 1995).

Techniques of development	**Herbicide resistant crops**
Traditional Selection	triazine-resistant canola
Seed Mutagenesis	terbutryn-resistant wheat sulfonyl urea-resistant soybean imidazolinone-resistant wheat

Cell Selection	sulfonyl urea-resistant canola atrazine-resistance in soybean
Genetic Engineering	sulfonyl urea-resistance - cotton Phosphinotricin (basta) resistance (rice, canola) glyphosate (Roundup) resistance (cotton, soybeans, maize, wheat) bromoxynil-resistant (cotton, subclover) 2,4-D resistant cotton

Most classes of herbicides exert their effect on a single enzyme which catalyses a key metabolic reaction in the plant.

Herbicides work by affecting a single enzyme, which causes a metabolic change in the plant. There are three methods by which a plant can convey herbicide resistance (OCDE, 1999):

1. Producing an enzyme which detoxified the enzyme
2. Producing an altered target enzyme which is not affected by the herbicide
3. Producing physical or physiological barriers to the uptake of the herbicide

Plants have been genetically engineered to be tolerant of a wide variety of herbicides. A variety of herbicide tolerant plants exists or is currently being developed for herbicide resistance or for use as selectable markers to identify transformed plants. Herbicide tolerance plants that has reached field-testing stages in the U.S. are listed in Table 2.

Table 2: Herbicides and herbicide-tolerant cultivars (adapted from Snow et. al, 1997)

Herbicide	**Herbicide-tolerant plant**
Butricil	Cotton, potato, tobacco
Phosphoinothiricin	Alfalfa, Arabidopsis, barley, beet, corn, creeping bentgrass, melon, peanut, poplar, rapeseed, rice, soybean, sugar cane, sweet potato, tobacco, tomato, wheat
Sulfonylurea	Corn, cotton, grape, rapeseed, tobacco, tomato

Introgression of herbicide resistance from conventionally bread crops into weeds was first reported in 1970 and since has been increasing at an exponential. Herbicide resistance to 17 classes of herbicide chemicals has been exhibited by biotypes of 172 weed species. Some major classes of herbicides to which weed resistance has been documented are shown in table 3:

Table 3: Herbicide resistance (adapted from Prather *et al*., 2000)

Type of resistance	**Number of resistant weed species (in the world)**
ACCase inhibitors	26
ALS inhibitors	63
Triazines	64
Ureas, amides	20
bipyridiliums	25
glycines	3
dinitroanilines	9
synthetic auxins	20

As a result, a number of herbicides have lost their agronomic usefulness.

Herbicide resistance can be prevented or delayed by

- herbicide rotation,
- monitoring after herbicide application,
- non-chemical control techniques,
- short residual herbicides
- certified seeds,
- clean equipment.

Herbicide resistant weeds can be managed by:

- Herbicide rotation
- Fallow tillage

- Close cultivation
- Prevention of weed seed spread through the use of clean equipment,
- Monitoring the initial evolution of resistance by recognizing patterns of weed escapes typical of resistant plants,
- Control of weeds suspected of herbicide resistance before they can produce seeds.

Unit 5.6.1. Advantages of herbicide resistant cultivars

Several advantages can be identified for the utilization of these new cultivars and these include:

(i) increased options - the availability of an extra herbicide option is extremely valuable. In some cases it simply allows the crop to be grown and in others it provides an alternate mode of action for the management of herbicide resistance development in the weed population;

(ii) more flexibility - the increased options provide greater flexibility in terms of crop rotations and the ability to respond quickly to market opportunities;

(iii) increased safety - where safer chemicals are able to be used, the risks to personal safety are clearly reduced. There may also be advantages where the chemical involved is environmentally benign or is used in very low concentrations;

(iv) reduced crop/pasture damage - in some cases herbicides do inflict damage to the crop or pasture in addition to the weeds. By having the resistance character in the productive plant the chances of yield-depressing damage may be averted. This is exemplified by the use of bromoxynil in subterranean clover where damage to the pasture can be significant, depending on the timing of the application;

(v) simplicity - in many cases the production system can be simplified - one application can replace two applications or two herbicides. We need to be aware, however, that in nature, simple means unstable and we need to think of the system in holistic and not marginal terms.

Unit 5.6.2. Concerns

Whilst the emergence of these cultivars is attractive in many cases, there is a number of aspects that should not be ignored:

(i) further development of resistance - The risk is that transgenic crops increase the reliance on a few herbicides and accelerate the selection pressure on weeds to evolve resistant biotypes. Herbicide resistant weeds can also appear via gene transfer from crop to weedy relatives.

Another problem of herbicide resistance now and for the future will involve weeds which exhibit multiple herbicide resistance. Unknown in plants until recently the phenomenon of multiple resistance is defined as the expression (within individuals or populations) of more than one resistance mechanism. Multiple resistant plants may possess from two to many distinct resistance mechanisms and may exhibit resistance to a few or many herbicides. Double and triple herbicide resistance plants have been found in canola fields in Canada in 1997 (Thomas, 2001; Westwood, 2001; Hall et al., 2000).

(ii) self-sown crop seed - these Herbicide Resistant crops will often germinate at the following season break where seed has been carried over from the previous harvest. Where they have resistance to herbicides, then clearly they will not be controlled by the application of that herbicide. Thus, for example in the case of glyphosate resistance, application of Roundup to provide a weed-free seedbed will be somewhat less successful where self-sown crop plants are present;

(iii) resistant escapes - in some cases, the opportunity exists for the resistant genes to escape. In the case of canola, for example, the ability exists through cross pollination to transfer the resistance character to related species (for example, wild radish, wild turnip) ultimately defeating the purpose for which the HR cultivar was developed;

(iv) abandonment of integrated weed management - as previously described, there will always be the temptation to forget the application of the principles of integrated weed management because of the increased simplicity of the system. Such temptation

needs to be avoided in order that we preserve the lives of both the cultivars and the herbicide;

(v) impact on conservation farming - this is particularly important when considering the impact of glyphosate-resistant crops. Roundup is an essential part of the conservation farming system and we need to preserve its role. Resistance buildup by weeds to this herbicide threatens our modern, soil-conserving practices of reduced tillage and we do not want to revert to the traditional ways of intensive cultivation. Resistance to glyphosate is now with us and we need to proceed with caution.

Unit 5.7. Transgenic insects

The application of gene technology to arthropods is not as advanced as that to crop plants but there are various research programmes in this area that are beginning to lead to products for which biosafety issues will have to be considered.

There are several breeding programmes for both the enhancement of beneficial insects and for producing material for pest control approaches. For instance, silkworms have been bred for many years to improve stock and there are breeding programmes for developing resistance of bees to diseases and pests and other characters. Pest resistance traits can be introduced into arthropods that are used for biological control.

A major way of controlling pest insects is by introducing sterile males into the natural population; this is termed the sterile insect release method (SIRM). This has been used mainly for pests of animals and for animal disease vectors but has equal applicability to some plant pests. In this method large numbers of males are rendered sterile by irradiation and complete with the natural fertile males. However, the method has various problems, one of the main ones being obtaining insect populations that are primarily male. The application of recombinant DNA technology to make the population predominantly male would help in overcoming these problems.

Unit 5.8. GM ARTHROPODS

As noted above, there are various research programmes aimed at genetically modifying insects. These include improving beneficial insects, conferring pesticide resistance onto biocontrol insects, improving insect viruses for insect control, producing mainly male progeny for SIRM, providing molecular markers to distinguish sterile from wild insects and making disease vectors unable to transmit diseases. There are two basic approaches to this, one to directly modify the insect itself and the other to modify symbionts that live within the insect.

There are two basic strategies to directly modify the insect itself. For the transgene to be expressed in all the insect cells and to be passed to subsequent generations, the germline (egg or sperm) cells have to be transformed. Several techniques such as microinjection or biolistics can be used to introduce the transgene into the egg or sperm cells. The main vectors used for this technique are transposable elements. Alternatively, the transgene can be introduced using a viral vector. For most viral vectors the transgene is only expressed in that generation of the insect and is not passed to the progeny (termed transient expression).

Most insects (and other arthropods) contain bacterial symbionts that are maternally transmitted. The rationale behind the second approach to genetically modifying insects is to transform the symbiotic bacteria, which can then release the transgene product into the insect haemocoel (blood). Microinjection using very fine glass capillary needles is used to reintroduce the modified symbionts back into the insect.

Most of the studies on transgenic insect have been attempts to control major human diseases such as malaria or dengue. Various GM strategies are being actively worked on to effect this control; these include sterile male production by inserting a dominant female specific factor, producing plasmodium-refractory strains (plasmodium is the organism that causes malaria) of the *Anopheles* mosquito and using two unlinked lethal genes each linked to a suppressor of the other lethal gene.

Unit 5.9: BIOSAFETY REGULATION

In 2006 there are some of the first applications to the biosafety regulatory authorities for release of GM insects. Thus these are early stages of developing biosafety regulatory structures for the release of such products. Because of the mobility of insects many of these questions are ecological and some differ from those considered for release of transgenic plants. These biosafety considerations are being built into the research programmes on developing transgenic insects. Although the current focus is on insects that impact with humans it is very likely that there will be consideration given soon to insects involved with the transmission of plant diseases; the biosafety considerations for these will be very similar to those for human disease vectors. Furthermore, the approach described above of rendering a virus vector incapable of transmitting the disease will have several biosafety advantages. It does not involve human and animal health considerations of transgenic crops or biodiversity issues of killing insects.

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MODULE 6

IMPROVEMENT OF CROP YIELDS, NITROGEN FIXATION, NUTRITIONAL QUALITY OF FOOD AND FEED, AND CHEMICAL COMPOSITION

UNIT 6.1. NITROGEN FIXATION

Role of nitrogen in the biosphere

The growth of all organisms depends on the availability of mineral nutrients, and none is more important than nitrogen, which is required in large amounts as an essential component of proteins, nucleic acids and other cellular constituents. There is an abundant supply of nitrogen in the earth's atmosphere - nearly 79% in the form of N_2 gas. However, N_2 is unavailable for use by most organisms because there is a triple

bond between the two nitrogen atoms, making the molecule almost inert. In order for nitrogen to be used for growth it must be "fixed" (combined) in the form of ammonium (NH_4) or nitrate (NO_3) ions. The weathering of rocks releases these ions so slowly that it has a neglible effect on the availability of fixed nitrogen. So, nitrogen is often the limiting factor for growth and biomass production in all environments where there is suitable climate and availability of water to support life.

Microorganisms have a central role in almost all aspects of nitrogen availability and thus for life support on earth:

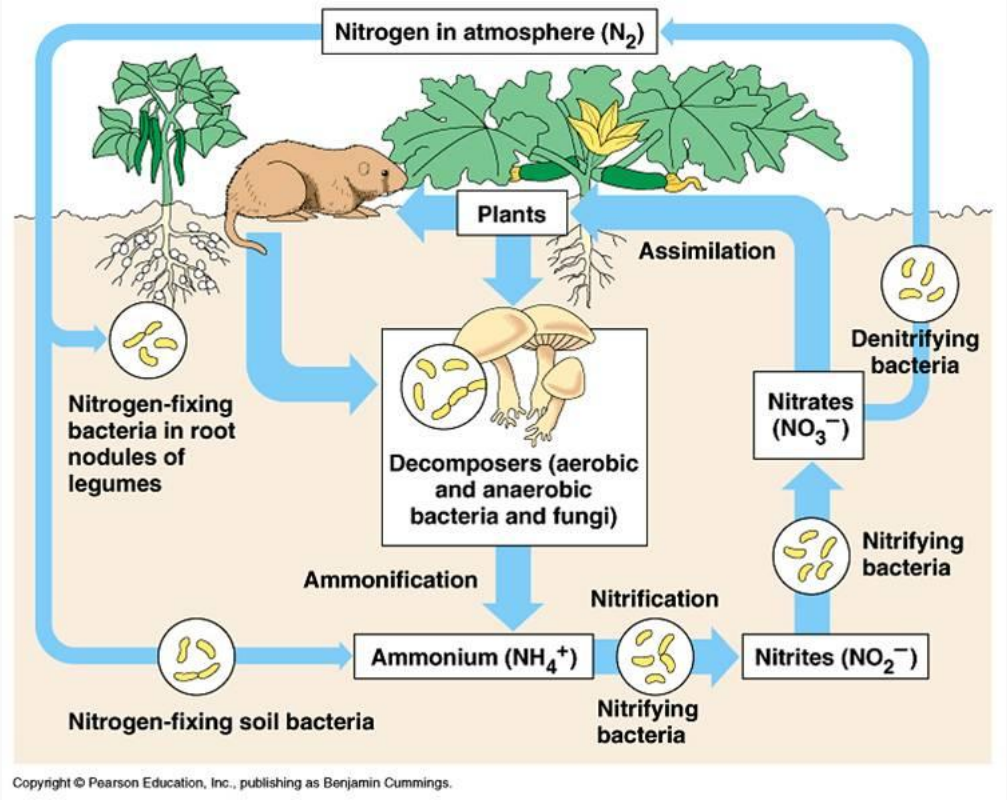
- some bacteria can convert N_2 into ammonia by the process termed **nitrogen fixation**; these bacteria are either free-living or form symbiotic associations with plants or other organisms (e.g. termites, protozoa)
- other bacteria bring about transformations of ammonia to nitrate, and of nitrate to N_2 or other nitrogen gases
- Many bacteria and fungi degrade organic matter, releasing fixed nitrogen for reuse by other organisms.

All these processes contribute to the **nitrogen cycle**.

Nitrogen fixation: is the natural process, either biological or abiotic, by which nitrogen (N_2) in the atmosphere is converted into ammonia (NH_3). This process is essential for life because fixed nitrogen is required to biosynthesize the basic building blocks of life, e.g. nucleotides for DNA and RNA and amino acids for proteins. Nitrogen fixation also refers to other biological conversions of nitrogen, such as its conversion to nitrogen dioxide.

Microorganisms that fix nitrogen are bacteria called diazotrophs. Some higher plants, and some animals (termites), have formed associations (symbioses) with diazotrophs. Nitrogen fixation also occurs as a result of non-biological processes. These include lightning, the industrial Haber-Bosch Process, and combustion Biological nitrogen fixation was discovered by the German agronomist Hermann Hellriegel and Dutch microbiologist Martinus Beijerinck.

Biological nitrogen fixation



Schematic representation of the nitrogen cycle. Abiotic nitrogen fixation has been omitted.

Biological nitrogen fixation (**BNF**) occurs when atmospheric nitrogen is converted to ammonia by an enzyme called nitrogenase. The reaction for BNF is:

$$N_2 + 8\ H^+ + 8\ e^- \rightarrow 2\ NH_3 + H_2$$

The process is coupled to the hydrolysis of 16 equivalents of ATP and is accompanied by the co-formation of one molecule of H_2. In free-living diazotrophs, the nitrogenase-generated ammonium is assimilated into glutamate through the glutamine synthetase/glutamate synthase pathway.

Enzymes responsible for nitrogenase action are very susceptible to destruction by oxygen. (In fact, many bacteria cease production of the enzyme in the presence of oxygen). Many nitrogen-fixing organisms exist only in anaerobic conditions, respiring to draw down oxygen levels, or binding the oxygen with a protein such as Leghemoglobin.

Microorganisms that fix nitrogen (diazotrophs)

- Cyanobacteria
- *Azotobacteraceae*
- *Rhizobia*
- *Frankia*

Nitrogen fixation by rhizobia and frankia

Rhizobia are Gram-negative with the ability to establish a N_2-fixing symbiosis on legume roots and on the stems of some aquatic legumes. During this interaction bacteroids, as rhizobia are called in the symbiotic state, are contained in intracellular compartments within a specialized organ, the nodule, where they fix N_2. Similarly, Frankia, Gram-positive soil bacteria induce the formation of nitrogen-fixing nodules in actinorhizal plants.

Nitrogen fixation by cyanobacteria

Cyanobacteria inhabit nearly all illuminated environments on Earth and play key roles in the carbon and nitrogen cycle of the biosphere. In general, cyanobacteria are able to utilize a variety of inorganic and organic sources of combined nitrogen, like nitrate, nitrite, ammonium, urea, or some amino acids. Several cyanobacterial strains are also capable of diazotrophic growth. Genome sequencing has provided a large amount of information on the genetic basis of nitrogen metabolism and its control in different cyanobacteria. Comparative genomics, together with functional studies, has led to a significant advance in this field over the past years. 2-Oxoglutarate has turned out to be the central signaling molecule reflecting the carbon/nitrogen balance of cyanobacteria. Central players of nitrogen control are the global transcriptional factor NtcA, which controls the expression of many genes involved in nitrogen metabolism, as well as the P_{II} signaling protein, which fine-tunes cellular activities in response to changing C/N conditions. These two proteins are sensors of the cellular 2-oxoglutarate level and have been conserved

in all cyanobacteria. In contrast, the adaptation to nitrogen starvation involves heterogeneous responses in different strains.[4]Nitrogen fixation by cyanobacteria in coral reefs can fix twice the amount of nitrogen than on land–around 1.8 kg of nitrogen is fixed per hectare per day.

Symbiotic nitrogen fixation

1. Legume symbioses

The most familiar examples of nitrogen-fixing symbioses are the **root nodules of legumes** (peas, beans, clover, etc.).

Part of a clover root system bearing naturally occurring nodules of *Rhizobium*. Each nodule is about 2-3 mm long.

Clover root nodules at higher magnification, showing two partly crushed nodules (arrowheads) with pink-coloured contents. This colour is caused by the presence of the pigment **leghaemoglobin** - a unique metabolite of this type of symbiosis. Leghaemoglobin is found only in the nodules and is not produced by either the bacterium or the plant when grown alone.

In these leguminous associations the bacteria usually are *Rhizobium* species, but the root nodules of soybeans, chickpea and some other legumes are formed by small-celled rhizobia termed *Bradyrhizobium*. Nodules on some tropical leguminous plants are formed by yet other genera. In all cases the bacteria "invade" the plant and cause the formation of a nodule by inducing localised proliferation of the plant host cells. Yet the bacteria always remain separated from the host cytoplasm by being enclosed in a membrane - a necessary feature in symbioses (see the image below).

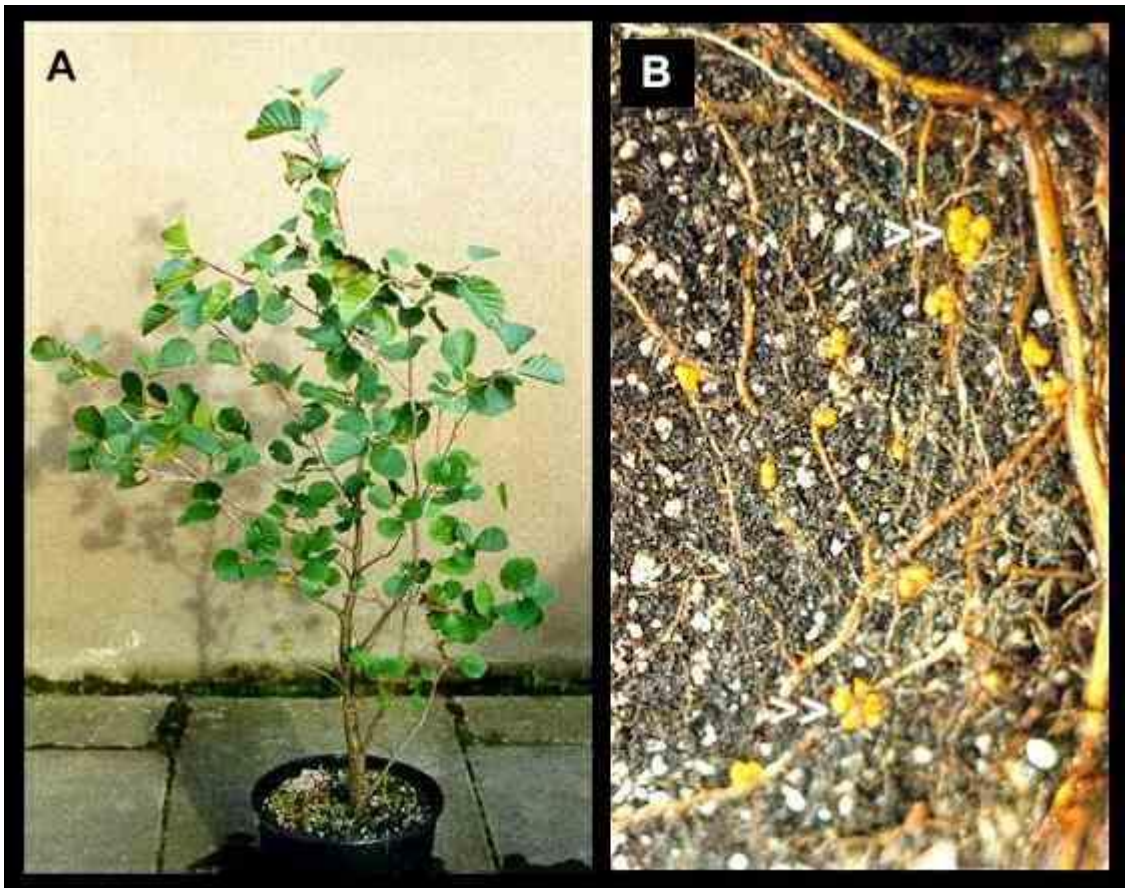
Part of a crushed root nodule of a pea plant, showing four root cells containing colonies of *Rhizobium*. The nuclei (**n**) of two root cells are shown; **cw** indicates the cell wall that separates two plant cells. Although it cannot be seen clearly in this image, the bacteria occur in clusters which are enclosed in membranes, separating them from the cytoplasm of the plant cells.

In nodules where nitrogen-fixation is occurring, the plant tissues contain the oxygen-scavenging molecule, **leghaemoglobin**(serving the same function as the oxygen-carrying haemoglobin in blood). The function of this molecule in nodules is to reduce the amount of free oxygen, and thereby to protect the nitrogen-fixing enzyme **nitrogenase**, which is irreversibly inactivated by oxygen.

2. Associations with *Frankia*

Frankia is a genus of the bacterial group termed **actinomycetes** - filamentous bacteria that are noted for their production of air-borne spores. Included in this group are the common soil-dwelling *Streptomyces* species which produce many of the antibiotics used in medicine (see *Streptomyces*). *Frankia* species are slow-growing in culture, and require specialised media, suggesting that they are specialised symbionts. They form nitrogen-fixing root nodules (sometimes called actinorhizae) with several woody

plants of different families, such as alder (*Alnus* species), sea buckthorn (*Hippophae rhamnoides*, which is common in sand-dune environments) and *Casuarina* (a Mediterranean tree genus). Figure A (below) shows a young alder tree (*Alnus glutinosa*) growing in a plant pot, and Figure B shows part of the root system of this tree, bearing the orange-yellow coloured nodules (arrowheads) containing *Frankia.*



Alder and the other woody hosts of *Frankia* are typical pioneer species that invade nutrient-poor soils. These plants probably benefit from the nitrogen-fixing association, while supplying the bacterial symbiont with photosynthetic products.

3. Cyanobacterial associations

The photosynthetic cyanobacteria often live as free-living organisms in pioneer habitats such as desert soils (seecyanobacteria) or as symbionts with lichens in other pioneer habitats. They also form symbiotic associations with other organisms such as the water fern *Azolla*, and cycads.The association with *Azolla*, where cyanobacteria

(*Anabaena azollae*) are harboured in the leaves, has sometimes been shown to be important for nitrogen inputs in rice paddies, especially if the fern is allowed to grow and then ploughed into the soil to release nitrogen before the rice crop is sown. A symbiotic association of cyanobacteria with cycads is shown below. The first image shows a pot-grown plant. The second image shows a close-up of the soil surface in this pot. Short, club-shaped, branching roots have grown into the aerial environment. These aerial roots contain a nitrogen-fixing cyanobacterial symbiont.

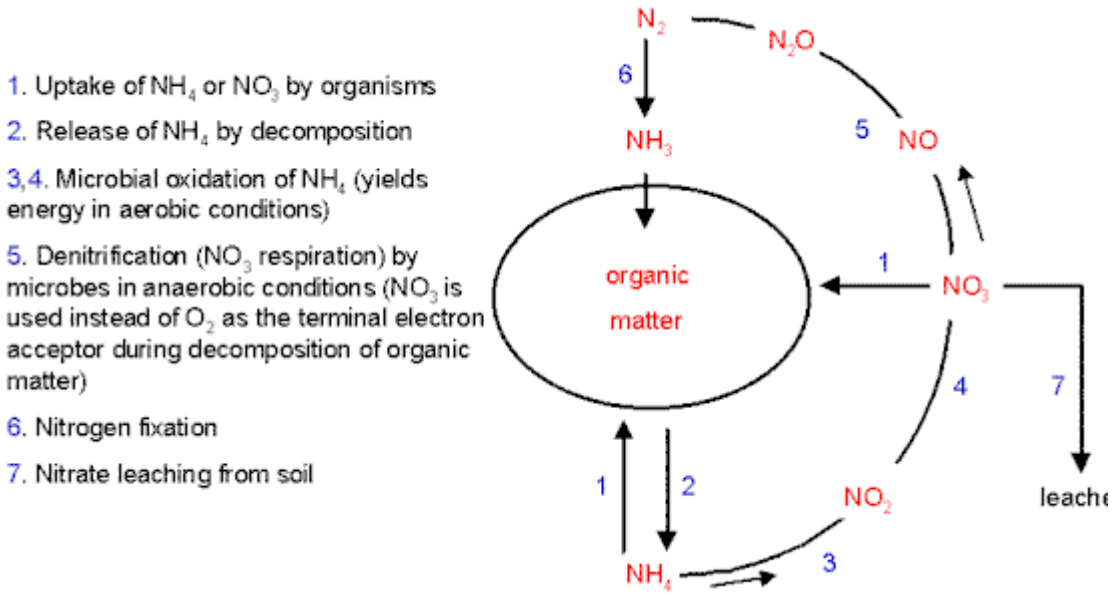
In addition to these intimate and specialised symbiotic associations, there are several free-living nitrogen-fixing bacteria that grow in close association with plants. For example, *Azospirillum* species have been shown to fix nitrogen when growing in the root zone (rhizosphere) or tropical grasses, and even of maize plants in field conditions. Similarly, *Azotobacter* species can fix nitrogen in the rhizosphere of several plants. In both cases the bacteria grow at the expense of sugars and other nutrients that leak from the roots. However, these bacteria can make only a small contribution to the nitrogen nutrition of the plant, because nitrogen-fixation is an energy-expensive process, and large amounts of organic nutrients are not continuously available to microbes in the rhizosphere.

This limitation may not apply to the bacteria that live in root nodules or other intimate symbiotic associations with plants. It has been estimated that nitrogen fixation in the nodules of clover roots or other leguminous plants may consume as much as 20% of the total photosynthate.

Unit 6.2. The nitrogen cycle

The diagram below shows an overview of the nitrogen cycle in soil or aquatic environments. At any one time a large proportion of the total fixed nitrogen will be locked up in the biomass or in the dead remains of organisms (shown collectively as "organic matter"). So, the only nitrogen available to support new growth will be that which is supplied by nitrogen fixation from the atmosphere (pathway 6 in the

diagram) or by the release of ammonium or simple organic nitrogen compounds through the decomposition of organic matter (pathway 2). Some of other stages in this cycle are mediated by specialised groups of microorganisms and are explained below.



Unit 6.3. Nitrification

The term nitrification refers to the conversion of ammonium to nitrate (pathway 3-4). This is brought about by the **nitrifying bacteria**, which are specialised to gain their energy by oxidising ammonium, while using CO_2 as their source of carbon to synthesise organic compounds. Organisms of this sort are termed **chemoautotrophs** - they gain their energy by chemical oxidations (chemo-) and they are autotrophs (self-feeders) because they do not depend on pre-formed organic matter. In principle the oxidation of ammonium by these bacteria is no different from the way in which humans gain energy by oxidising sugars. Their use of CO_2 to produce organic matter is no different in principle from the behaviour of plants.

The nitrifying bacteria are found in most soils and waters of moderate pH, but are not active in highly acidic soils. They almost always are found as mixed-species communities (termed **consortia**) because some of them - e.g. *Nitrosomonas* species - are specialised to convert ammonium to nitrite (NO_2^-) while others - e.g. *Nitrobacter* species - convert nitrite to nitrate (NO_3^-). In fact, the accumulation of

nitrite inhibits *Nitrosomonas*, so it depends on *Nitrobacter* to convert this to nitrate, whereas*Nitrobacter* depends on *Nitrosomonas* to generate nitrite.

The nitrifying bacteria have some important environmental consequences, because they are so common that most of the ammonium in oxygenated soil or natural waters is readily converted to nitrate. Most plants and microorganisms can take up either nitrate or ammonium (arrows marked "1" in the diagram). However, process of nitrification has some undesirable consequences. The ammonium ion (NH_4^+) has a positive charge and so is readily adsorbed onto the negatively charged clay colloids and soil organic matter, preventing it from being washed out of the soil by rainfall. In contrast, the negatively charged nitrate ion is not held on soil particles and so can be washed down the soil profile - the process termed leaching (arrow marked 7 in the diagram). In this way, valuable nitrogen can be lost from the soil, reducing the soil fertility. The nitrates can then accumulate in groundwater, and ultimately in drinking water. There are strict regulations governing the amount of nitrate that can be present in drinking water, because nitrates can be reduced to highly reactive nitrites by microorganisms in the anaerobic conditions of the gut. Nitrites are absorbed from the gut and bind to haemoglobin, reducing its oxygen-carrying capacity. In young babies this can lead to respiratory distress - the condition known as "blue baby syndrome". Nitrite in the gut also can react with amino compounds, forming highly carcinogenic nitrosamines.

Unit 6.4 Denitrification

Denitrification refers to the process in which nitrate is converted to gaseous compounds (nitric oxide, nitrous oxide and N_2) by microorganisms. The sequence usually involves the production of nitrite (NO_2^-) as an intermediate step is shown as "5" in the diagram above. Several types of bacteria perform this conversion when growing on organic matter in anaerobic conditions. Because of the lack of oxygen for normal aerobic respiration, they use nitrate in place of oxygen as the terminal electron acceptor. This is termed anaerobic respiration and can be illustrated as follows:

In aerobic respiration (as in humans), organic molecules are oxidised to obtain energy,

while oxygen is reduced to water:

$C_6H_{12}O_6 + 6\ O_2 = 6\ CO_2 + 6\ H_2O$ + energy

In the absence of oxygen, any reducible substance such as nitrate (NO_3^-) could serve the same role and be reduced to nitrite, nitric oxide, nitrous oxide or N_2.

Thus, the conditions in which we find denitrifying organisms are characterised by (1) a supply of oxidisable organic matter, and (2) absence of oxygen but availability of reducible nitrogen sources. A mixture of gaseous nitrogen products is often produced because of the stepwise use of nitrate, nitrite, nitric oxide and nitrous oxide as electron acceptors in anaerobic respiration. The common denitrifying bacteria include several species of *Pseudomonas*, *Alkaligenes* and *Bacillus*. Their activities result in substantial losses of nitrogen into the atmosphere, roughly balancing the amount of nitrogen fixation that occurs each year.

Root nodule symbioses

Legume family

Plants that contribute to nitrogen fixation include the legume family – Fabaceae – with taxa such as clovers, soybeans, alfalfa, lupines, peanuts, and rooibos. They contain symbiotic bacteria called *Rhizobia* within nodules in their root systems, producing nitrogen compounds that help the plant to grow and compete with other plants. When the plant dies, the fixed nitrogen is released; making it available to other plants and this helps to fertilize the soil. The great majority of legumes have this association, but a few genera (e.g., *Styphnolobium*) do not. In many traditional and organic farming practices, fields are rotated through various types of crops, which usually includes one consisting mainly or entirely of clover or buckwheat (non-legume family *Polygonaceae*), which are often referred to as "green manure."

Inga alley farming relies on the leguminous genus, Inga a small tropical, tough-leaved, nitrogen-fixing tree.

Non-leguminous

A sectioned alder tree root nodule.

A whole alder tree root nodule.

Although by far the majority plants able to form nitrogen-fixing root nodules are in the legume family Fabaceae, there are a few exceptions:

- Parasponia, a tropical Celtidaceae also able to interact with rhizobia and form nitrogen-fixing nodules
- Actinorhizal plants such as alder and bayberry, that can also forms nitrogen-fixing nodules, thanks to a symbiotic association with Frankia bacteria. These plants belong to 25 genera distributed among 8 plant families. The ability to fix nitrogen is far from universally present in these families. For instance, of 122 genus in the Rosaceae, only 4 genera are capable of fixing nitrogen. All these families belong to the orders Cucurbitales, Fagales, and Rosales, which together with the Fabales form a clade of eurosids. In this clade, Fabales were the first lineage to branch off; thus, the ability to fix nitrogen may be plesiomorphic and subsequently lost in most descendants of the original nitrogen-fixing plant; however, it may be that the basic genetic and physiological requirements were present in an incipient state

in the last common ancestors of all these plants, but only evolved to full function in some of them:

Coriariaceae: *Coriaria* .....

Datiscaceae: *Datisca*

Elaeagnaceae:

Elaeagnus(silverberries)

Hippophae (sea-buckthorns)

Shepherdia(buffaloberries)

There are also several nitrogen-fixing symbiotic associations thatinvolve cyanobacteria (such as *Nostoc*):

- Some lichens such as *Lobaria* and *Peltigera*
- Mosquito fern (*Azolla* species)
- Cycads
- *Gunnera*

Chemical nitrogen fixation

Haber process

Nitrogen can also be artificially fixed as ammonia for use in fertilizers, explosives, or in other products. The most common method is the Haber process. Artificial fertilizer production is now the largest source of human-produced fixed nitrogen in the Earth's ecosystem.

The Haber process requires high pressures (around 200 atm) and high temperatures (at least 400 °C), routine conditions for industrial catalysis. This highly efficient process uses natural gas as a hydrogen source and air as a nitrogen source.

Dinitrogen complexes

Much research has been conducted on the discovery of catalysts for nitrogen fixation, often with the goal of reducing the energy required for this conversion.

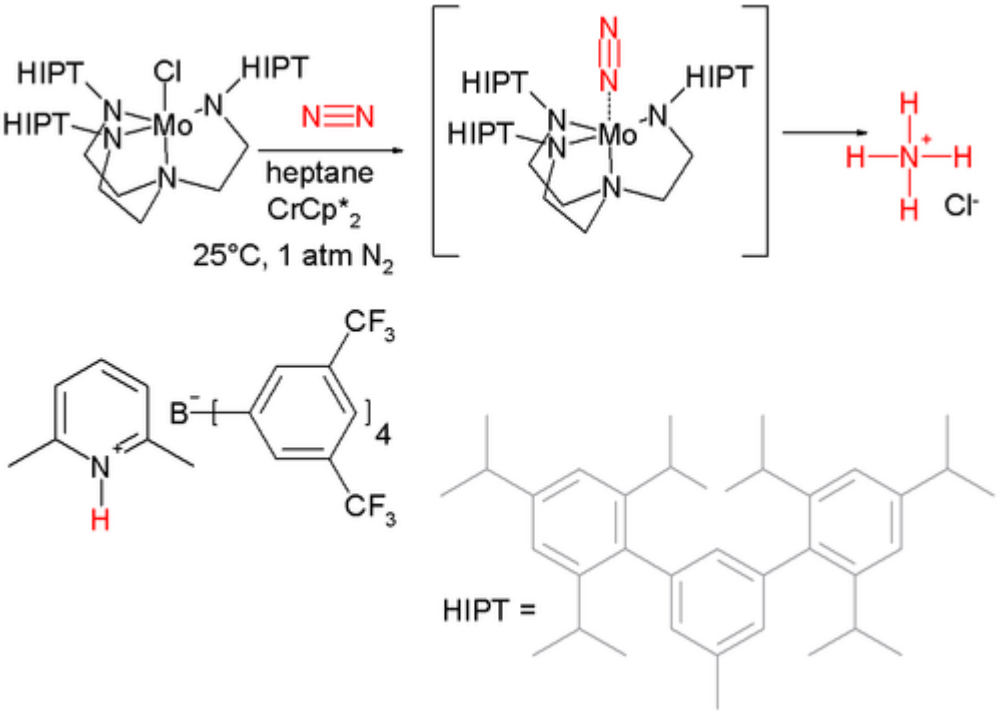
However, such research has thus far failed to even approach the efficiency and ease of the Haber process. Many compounds react with atmospheric nitrogen under ambient conditions. For example, lithium metal converts to lithium nitride under an atmosphere of nitrogen. Treatment of the resulting nitride gives ammonia.

The first dinitrogen complex was reported in 1965 based on ammonia coordinated to ruthenium ($[Ru(NH_3)_5(N_2)]^{2+}$).[10] Research in chemical fixation from then on focused on transition metal complexes. Since then, a large number of transition metal compounds that contain dinitrogen as a legend have been discovered. The dinitrogen ligand can either be bound to a single metal or bridge two (or more) metals. The coordination chemistry of dinitrogen is complex and currently under intense investigation. This research may lead to new ways of using dinitrogen in synthesis and on an industrial scale.

Ambient nitrogen reduction

Catalytic chemical nitrogen fixation at temperatures considerably lower than the Haber process is an ongoing scientific endeavor. Nitrogen was successfully converted to ammonia and hydrazine by Alexander E. Shilov in 1970. The first example of homolytic cleavage of dinitrogen under mild conditions was published in 1995. Two equivalents of a molybdenum complex reacted with one equivalent of dinitrogen, creating a triple bonded MoN complex.] Since then, this triple bounded complex has been used to make nitriles.

The first catalytic system converting nitrogen to ammonia at room temperature and pressure was discovered in 2003 and is based on another molybdenum compound, a proton source, and a strong reducing agent. However, this catalytic reduction fixates only a few nitrogen molecules.



In 2011 Arashiba et al. reported another system with a catalyst again based on molybdenum but with a diphosphorus pincer ligand.[19]

- Birkeland–Eyde process
- Haber process
- Denitrification
- George Washington Carver
- Nif gene
- Nitrification
- Nitrogen cycle
- Nitrogen deficiency
- Nitrogenase
- Push–pull technology

References

1. Postgate, J (1998). *Nitrogen Fixation, 3rd Edition*. Cambridge University Press, Cambridge UK.
2. http://helios.bto.ed.ac.uk/bto/microbes/nitrogen.htm

3. Moir, JWB (editor) (2011). *Nitrogen cycling in bacteria: Molecular analysis*. Caister Academic Press. ISBN 978-1-904455-86-8.
4. Herrero A and Flores E (editor). (2008). *The Cyanobacteria: Molecular Biology, Genomics and Evolution* (1st ed.). Caister Academic Press. ISBN 978-1-904455-15-8. [1].
5. Smil, V (2000). *Cycles of Life*. Scientific American Library.
6. Elkan, Daniel. *Slash-and-burn farming has become a major threat to the world's rainforest* The Guardian 21 April 2004
7. Op den Camp, Rik; *et al.*. "LysM-Type Mycorrhizal Receptor Recruited for Rhizobium Symbiosis in Nonlegume *Parasponia*".*Science* **331** (6019): 909–912. doi:10.1126/science.1198181.
8. Dawson, J. O. (2008). "Ecology of actinorhizal plants". *Nitrogen-fixing Actinorhizal Symbioses*. **6**. Springer. pp. 199–234.doi:10.1007/978-1-4020-3547-0_8.
9. http://www.epa.gov/watertrain/nitroabstr.html US Enivronmental Protection Agency: Human Alteration of the Global Nitrogen Cycle: Causes and Consequences by Peter M. Vitousek, Chair, John Aber, Robert W. Howarth, Gene E. Likens, Pamela A. Matson, David W. Schindler, William H. Schlesinger, and G. David Tilman
10. A. D. Allen, C. V. Senoff (1965). "Nitrogenopentammineruthenium(II) complexes". *Journal of the Chemical Society, Chemical Communications* (24): 621.doi:10.1039/C19650000621.
11. *Catalytic reduction of molecular nitrogen in solutions* A.E. Shilov Russian Chemical Bulletin Volume 52, Number 12, 2555-2562,doi:10.1023/B:RUCB.0000019873.81002.60
12. *Reduction of dinitrogen* Richard R. Schrock PNAS November 14, 2006 vol. 103 no. 46 17087 doi:10.1073/pnas.0603633103
13. *Dinitrogen Cleavage by a Three-Coordinate Molybdenum(III) Complex* Catalina E. Laplaza and Christopher C. Cummins Science 12 May 1995: 861-863.10.1126/science.268.5212.861

14. *A Cycle for Organic Nitrile Synthesis via Dinitrogen Cleavage*John J. Curley, Emma L. Sceats, and Christopher C. Cummins J. Am. Chem. Soc., 2006, 128 (43), pp 14036–14037doi:10.1021/ja066090a

15. *Synthesis and Reactions of Molybdenum Triamidoamine Complexes Containing Hexaisopropylterphenyl Substituents*Dmitry V. Yandulov, Richard R. Schrock, Arnold L. Rheingold, Christopher Ceccarelli, and William M. Davis Inorg. Chem.; **2003**; 42(3) pp 796–813; (Article) doi:10.1021/ic020505l

16. *Catalytic Reduction of Dinitrogen to Ammonia at a Single Molybdenum Center* Dmitry V. Yandulov and Richard R. SchrockScience 4 July **2003**: Vol. 301. no. 5629, pp. 76–78doi:10.1126/science.1085326

17. The catalyst is based on molybdenum(V) chloride and tris(2-aminoethyl)amine substituted with three very bulky hexa-isopropylterphenyl (HIPT) groups. Nitrogen adds end-on to the molybdenum atom, and the bulky HIPT substituents prevent the formation of the stable and nonreactive Mo-N=N-Mo dimer, and the nitrogen is reduced in an isolated pocket. The proton donor is apyridinium cation, which is accompanied by a tetraborate counter ion. The reducing agent is decamethylchromocene. All ammonia formed is collected as the HCl salt by trapping the distillate with a HCl solution

18. Note also that, although the dinitrogen complex is shown in brackets, this species can be isolated and characterized. Here the brackets do not indicate that the intermediate is not observed.

19. *A molybdenum complex bearing PNP-type pincer ligands leads to the catalytic reduction of dinitrogen into ammonia* Kazuya Arashiba, Yoshihiro Miyake Yoshiaki

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MODULE 7

BIOFERTILIZERS

Bio Fertilizer is a Natural organic fertilizer known that helps to provide all the nutrients required by the plants and helps to increase the quality of the the soil with a natural microorganism enviroment.

Our fertilizers are 100% natural organic material from the nutrient-rich lands of Costa Rica, that could be easily applied with water over your plantations on any stage, or use it as a base for creating a potent and concentrated natural organic liquid fertilizers that could be used in many different growing mediums with excellent production results for organic farming, industrial and home applications such as gardening.

BioFertilizers are the most advanced bio technology necessary to support developing organic agriculture, sustainable agriculture, green agriculture and non-pollution agriculture. This Bio-organic Fertilizer can increase the output, improve the quality and it is responsible for agriculture environment. Today, It has been widely used with excellent results in all kinds of plants and several countries.

It is well known that the continue use and overuse of petrochemical based fertilizers and toxic pesticides have caused a detrimental effect to our soils, water supplies, foods, animals and even people. The Biological Grower is more sensible with sustainable approach and employs the resources of both science and nature to allow better results in his production. For centuries, peat moss has been recognized soil bacteria, fungi, earthworms, and other bio-organisms to enrich the soil to produce safe, nutritious and abundant crops. an excellent fertilizer sources. We have developed all natural organic fertilizers made with pure peat moss .

Biofertilizer Contains a wide range of naturally chelated plant nutrients and trace elements, carbohydrates, amino acids and other growth promoting substances. Kelp acts as a soil conditioner by stimulating microbial activity in the soil which results in improved air-water relationships in soil, improved fertility and makes

soil less prone to compaction and erosion. Organic Growers who use kelp in their regular fertility program report increases in yield, quality, shelf-life and resistance to environmental stresses such as drought, extreme heat, early frost, pest and disease problems.

This blend makes an excellent foliar fertilizer. Besides being a nutritionally complete fertilizer (containing even calcium), the nutrients are readily absorbed by the leaf. This is because the nitrogen in fish is in the form of amino acids which plants take in and use directly– unlike inorganic fertilizers in which the nitrogen needs to be converted into a usable form first. Additionally, because the micro-nutrients in the fish and in the kelp are in a naturally chelated form they are quickly and readily absorbed into the leaf surface. Foliar applications on a regular basis can increase the health, vigour and yield of plants due to this easily absorbed additional nutrition.

Organic fertilizers differ from chemicals fertilizers in that they feed your plants while adding organic material to the soil. Soils with lots of organic matter remain loose and airy, hold more moisture and nutrients, foster growth of soil organisms, and promote healthier plant root development. If only chemicals are added the soil gradually loses its organic matter and microbiotic activity. As organic matter is used up, the soil structure deteriorates, becoming compact, lifeless and less able to hold water and nutrients. This results in increased amounts of chemical fertilizers needed to feed plants. We also like organic fertilizers because they're made from renewable resources; chemicals are not.

The Bio Fertilizer, is a premium natural fertilizer composed just with certified organic ingredients special for nutrient-poor Western soils. This organic fertilizers is unequaled in its ability to nourish the beneficial micro-organisms in the soil greatly increasing the soil’s humus content and improving its ability to sustain and nurture healthy, more colorful plants. Use by the handful when planting individual plants, broadcast and mix it deeply into the soil when planting flower beds or spread it around established plants and scratch it into the soil. It is also excellent for use in vegetable gardens, container plantings and as a compost-pile activator

The Peat moss is a plant that could lives well in an environment poor in oxygen but when dies, it's decomposition could takes thousands of years, That property, give them special properties to the cells, making them much bigger and it that helps to KEEPS the water and nutrients that could be applied later to the plants.

Bio Fertilizer: The Best Economic Value: Proven, top-quality product. Stick with biofertilizer the one that always has and always will give you top quality and the best value immediately for your investment and much more profits at long term. Research winner. Growing trials conducted by an independent research center at a professional greenhouse compared various soils amended with peat, coir, compost and blends. The conclusion:

"Sphagnum peat can be considered the best overall performer as a soil amendment and substrate: it is homogenous, easy to handle and has shown the best growth results; all of this at a highly competitive price."

Unit 7.2. What is biofertilizer?

The name itself is self explanatory. The fertilizers are used to improve the fertility of the land using biological wastes, hence the term biofertilizers, and biological wastes do not contain any chemicals which are detrimental to the living soil. They are extremely beneficial in enriching the soil with those micro-organisms, which produce organic nutrients for the soil and help combat diseases. The farm produce does not contain traces of hazardous and poisonous materials. Thus those products are accepted across the world as Organic ones. Hence for organic farming the use of biofertilizers is mandatory.

Galaxy of Biofertilizers

Phospho: It releases insoluble phosphorus in soil and fix this phosphorus in clay minerals which is of great significance in agriculture.

Rhizo: Rhizo Bacterial plays a very important role in agriculture by inducing nitrogen fixings nodules on the root of legumes such as peas,beans clove and

alfalfa.

Azotobactor: Atmosphere contains 78% nitrogen which is a very important nutrient for plant growth. Azotobactor fixes the atmospheric nitrogen in the soil and make it available to the plants. It protects the roots from other pathogens present in the soil

Trichoderma: It is a non- pathogenic and eco-friendly product. The product is antagonistic hyper parasitic against different pathogens in the field and economically well established biocontrol agent

Composter: (Decomposing Culture): Composter breaks down any organic matter such as dead plants farm yard waste, cattle waste etc. thereby increasing the soil productivity.

Tricho-Card: Trichogramma is an efficient destroyer of eggs of many leaf and flower eaters, stems, fruit, shoot borers etc. It can be used in a variety of crops as well as in horticultural and ornamental plants, such as sugarcane,cotton,brinjal,tomato,corn,jawar,vegetables,citrus,paddy apple etc.

Vermi Compost: It is 100% pure eco-friendly organic fertilizer. This organic fertilizer has nitrogen phosphorus, potassium,organic carbon,sulphur,hormones,vitamins,enzymes and antibiotics which helps to improve the quality and quantity of yield. It is observed that due to continuous misuse of chemical fertiliser soil losses its fertility and gets salty day by day. To overcome such problems natural farming is the only remedy and Vermi compost is the best solution.

Biocompost: It is eco-friendly organic fertilizer which is prepared from the sugar industry waste material which is decomposed and enriched of with various plants and human friendly bacteria and fungi. Biocompost consists of nitrogen, phosphate solubilizing bacteria and various useful fungi like decomposing fungi, trichoderma viridea which protects the plants from various soil borne disease and also help to increase soil fertility which results to a good quality product to the farmers.

New technique in Biofertilizer

Generally biofertilizers are carrier based and always in powder form. The carrier is mostly lignite, which has high organic matter content, and it holds more than 200% water, which enhances the growth of the micro-organisms. Before use slurry is made which is applied to seed. This method is universal unless it has some difficulty in application to seed no other method is used.

Recently university of agricultural sciences, Bangalore has developed a method-dry complex fertilizer for direct soil application. This method consists of granules (1-2 mm) made from tank bed clay (TBC). These granules are baked at 2000C in a muffle furnace, which helps to sterilize the material and gives porosity to granules. The baked granules are soaked in a suspension of nitrogen fixing bacteria grown in a suitable medium overnight. The clay granules are air dried at room temperature under aseptic conditions. The granules contain more than a billion of bacteria per gram of granules. These granules are suitable for field application along with seeds and fertilizers in furrows. However, quantity of biofertilizer to be applied will be slightly higher than seed application.

TRIAL QUESTIONS

1. **What is biofertilizer?**

Biofertilizers are ready to use live formulates of such beneficial microorganisms which on application to seed, root or soil mobilize the availability of nutrients by their biological activity in particular, and help build up the micro-flora and in turn the soil health in general.

2. **Why should we use biofertilizers**?

With the introduction of green revolution technologies the modern agriculture is getting more and more dependent upon the steady supply of synthetic inputs (mainly fertilizers), which are products of fossil fuel (coal+ petroleum). Adverse

effects are being noticed due to the excessive and imbalanced use of these synthetic inputs. This situation has lead to identifying harmless inputs like biofertilizers. Use of such natural products like biofertilizers in crop cultivation will help in safeguarding the soil health and also the quality of crop products.

3. What are the benefits from using biofertilizers?

- Increase crop yield by 20-30%.
- Replace chemical nitrogen and phosphorus by 25%.
- Stimulate plant growth.
- Activate the soil biologically.
- Restore natural soil fertility.
- Provide protection against drought and some soil borne diseases.

4. What are the advantages of bio-fertilizers?

1. Cost effective.
2. Supplement to fertilizers.
3. Eco-friendly (Friendly with nature).
4. Reduces the costs towards fertilizers use, especially regarding nitrogen and phosphorus.

5. What types of biofertilizers are available?

1. For Nitrogen
    - Rhizobium for legume crops.
    - Azotobacter/Azospirillum for non legume crops.
    - Acetobacter for sugarcane only.
    - Blue –Green Algae (BGA) and Azolla for low land paddy.
2. For Phosphorous
    - Phosphatika for all crops to be applied with Rhizobium, Azotobacter, Azospirillum and Acetobacter
3. For enriched compost
    - Cellulolytic fungal culture

- Phosphotika and Azotobacter culture

6. What biofertilizers are recommended for crops?

- Rhizobium + Phosphotika at 200 gm each per 10 kg of seed as seed treatment are recommended for pulses such as pigeonpea, green gram, black gram, cowpea etc, groundnut and soybean.
- Azotobacter + Phosphotika at 200 gm each per 10 kg of seed as seed treatment are useful for wheat, sorghum, maize, cotton, mustard etc.
- For transplanted rice, the recommendation is to dip the roots of seedlings for 8 to 10 hours in a solution of Azospirillum + Phosphotika at 5 kg each per ha.

7. How biofertilizers are applied to crops?

1. Seed treatment:
   200 g of nitrogenous biofertilizer and 200 g of Phosphotika are suspended in 300-400 ml of water and mixed thoroughly. Ten kg seeds are treated with this paste and dried in shade. The treated seeds have to be sown as soon as possible.
2. Seedling root dip:
   For rice crop, a bed is made in the field and filled with water.
   Recommended biofertilizers are mixed in this water and the roots of seedlings are dipped for 8-10 hrs.
3. Soil treatment:
   4 kg each of the recommended biofertilizers are mixed in 200 kg of compost and kept overnight. This mixture is incorporated in the soil at the time of sowing or planting.

8. How could one get good response to biofertilizer application?

- Biofertilizer product must contain good effective strain in appropriate population and should be free from contaminating microorganisms.
- Select right combination of biofertilizers and use before expiry date.
- Use suggested method of application and apply at appropriate time as per

the information provided on the label.

- For seed treatment adequate adhesive should be used for better results.
- For problematic soils use corrective methods like lime or gypsum pelleting of seeds or correction of soil pH by use of lime.
- Ensure the supply of phosphorus and other nutrients.

9. What would be probable reasons for not getting response from the application of biofertilizers?

1. On account of quality of product
    - Use of ineffective strain.
    - Insufficient population of microorganisms.
    - High level of contaminants.
2. On account of inadequate storage facilities
    - May have been exposed to high temperature.
    - May have been stored in hostile conditions.
3. On account of usage
    - Not used by recommended method in appropriate doses.
    - Poor quality adhesive.
    - Used with strong doses of plant protection chemicals.
4. On account of soil and environment
    - High soil temperature or low soil moisture.
    - Acidity or alkalinity in soil.
    - Poor availability of phosphorous and molybdenum.
    - Presence of high native population or presence of bacteriophages.

10. What precautions one should take for using biofertilizers?

- Biofertilizer packets need to be stored in cool and dry place away from direct sunlight and heat.
- Right combinations of biofertilizers have to be used.
- As Rhizobium is crop specific, one should use for the specified crop only.
- Other chemicals should not be mixed with the biofertilizers.
- While purchasing one should ensure that each packet is provided with

necessary information like name of the product, name of the crop for which intended, name and address of the manufacturer, date of manufacture, date of expiry, batch number and instructions for use.

- The packet has to be used before its expiry, only for the specified crop and by the recommended method of application.
- Biofertilizers are live product and require care in the storage
- Both nitrogenous and phosphatic biofertilizers are to be used to get the best results.
- It is important to use biofertilizers along with chemical fertilizers and organic manures.
- Biofertilizers are not replacement of fertilizers but can supplement plant nutrient requirements.

11. Where can I get further information on biofertilizers?

You may visit the following Internet sites:

http://www.ikisan.com/links/up_riceBiofertilizers.shtml#top
http://www.entireindia.com/YellowPg/YpCatList.asp?s=1159&cnm=Biofertilizers
http://www.glsbiotech.com/products.htm#biofertilizers
http://www.us.erc.org/greenchannel/gc7/innovativebiotechnologicalproductsforagriculture.php www.suvash.com
http://www.kumarbuilders.com/bio.htm,

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MODULE 8

MOLECULAR MARKER

Adapted from Kumar, A. 2009.
http://www.science20.com/humboldt_fellow_and_science/molecular_markers

Due to the rapid developments in the field of molecular genetics, a variety of different techniques have emerged to analyze genetic variation during the last few decades (Whitkus et al. 1994; Karp et al. 1996, 1997a,b; Parker et al. 1998; Schlötterer 2004). These genetic markers may differ with respect to important features, such as genomic abundance, level of polymorphism detected, locus specificity, reproducibility, technical requirements and financial investment. No marker is superior to all others for a wide range of applications. The most appropriate genetic marker will depend on the specific application, the presumed level of polymorphism, the presence of sufficient technical facilities and know-how, time constraints and financial limitations.

A genetic marker is a gene or DNA sequence with a known location on a chromosome and associated with a particular gene or trait. It can be described as a variation, which may arise due to mutation or alteration in the genomic loci that can be observed. A genetic marker may be a short DNA sequence, such as a sequence surrounding a single base-pair change (single nucleotide polymorphism, SNP), or a long one, like minisatellites.

For many years, gene mapping was limited in most organisms by traditional genetic markers which include genes that encode easily observable characteristics such as blood types or seed shapes. The insufficient amount of these types of characteristics in several organisms limited the mapping efforts that could be done.

Some commonly used types of genetic markers are

- RFLP (or Restriction fragment length polymorphism)
- AFLP (or Amplified fragment length polymorphism)
- RAPD (or Random amplification of polymorphic DNA)
- VNTR (or Variable number tandem repeat)
- Microsatellite polymorphism
- SNP (or Single nucleotide polymorphism)
- STR (or Short tandem repeat)

• SFP (or Single feature polymorphism)
• DArT (or Diversity Arrays Technology)

They can be further categorized as dominant or co-dominant. Dominant markers allow for analyzing many loci at one time, e.g. RAPD. A primer amplifying a dominant marker could amplify at many loci in one sample of DNA with one PCR reaction. Co-dominant markers analyze one locus at a time. A primer amplifying a co-dominant marker would yield one targeted product.

1. Restriction fragment length polymorphism

In molecular biology, the term restriction fragment length polymorphism, or RFLP, (commonly pronounced "rif-lip") refers to a difference between two or more samples of homologous DNA molecules arising from differing locations of restriction sites, and to a related laboratory technique by which these segments can be distinguished. In RFLP analysis the DNA sample is broken into pieces (digested) by restriction enzymes and the resulting restriction fragments are separated according to their lengths by gel electrophoresis. Although now largely obsolete, RFLP analysis was the first DNA profiling technique cheap enough to see widespread application. In addition to genetic fingerprinting, RFLP was an important tool in genome mapping, localization of genes for genetic disorders, determination of risk for disease, and paternity testing.

Amplified fragment length polymorphism

Amplified Fragment Length Polymorphism PCR (or AFLP-PCR or just AFLP) is a PCR-based tool used in genetics research, DNA fingerprinting, and in the practice of genetic engineering. Developed in the early 1990's by Keygene[1], AFLP uses restriction enzymes to cut genomic DNA, followed by ligation of adaptors to the sticky ends of the restriction fragments. A subset of the restriction fragments are then amplified using primers complementary to the adaptor and part of the restriction site fragments (as described in detail below). The amplified fragments are visualized on

denaturing polyacrylamide gels either through autoradiography or fluorescence methodologies.

AFLP-PCR is a highly sensitive method for detecting polymorphisms in DNA. The technique was originally described by Vos and Zabeau in 1993. In detail, the procedure of this technique is divided into three steps:

1. Digestion of total cellular DNA with one or more restriction enzymes and ligation of restriction half-site specific adaptors to all restriction fragments.

2. Selective amplification of some of these fragments with two PCR primers that have corresponding adaptor and restriction site specific sequences.

3. Electrophoretic separation of amplicons on a gel matrix, followed by visualisation of the band pattern.

A variation on AFLP is cDNA-AFLP, which is used to quantify differences in gene expression levels.

Applications of AFLP:

The AFLP technology has the capability to detect various polymorphisms in different genomic regions simultaneously. It is also highly sensitive and reproducible. As a result, AFLP has become widely used for the identification of genetic variation in strains or closely related species of plants, fungi, animals, and bacteria. The AFLP technology has been used in criminal and paternity tests, in population genetics to determine slight differences within populations, and in linkage studies to generate maps for quantitative trait locus (QTL) analysis.

There are many advantages to AFLP when compared to other marker technologies including randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), and microsatellites. AFLP not only has higher reproducibility,

resolution, and sensitivity at the whole genome level compared to other techniques[4], but it also has the capability to amplify between 50 and 100 fragments at one time. In addition, no prior sequence information is needed for amplification (Meudt&Clarke 2007)[5]. As a result, AFLP has become extremely beneficial in the study of taxa including bacteria, fungi, and plants, where much is still unknown about the genomic makeup of various organisms.

RAPD:

RAPD (pronounced "rapid") stands for Random Amplification of Polymorphic DNA. It is a type of PCR reaction, but the segments of DNA that are amplified are random. The scientist performing RAPD creates several arbitrary, short primers (8-12 nucleotides), then proceeds with the PCR using a large template of genomic DNA, hoping that fragments will amplify. By resolving the resulting patterns, a semi-unique profile can be gleaned from a RAPD reaction.

No knowledge of the DNA sequence for the targeted gene is required, as the primers will bind somewhere in the sequence, but it is not certain exactly where. This makes the method popular for comparing the DNA of biological systems that have not had the attention of the scientific community, or in a system in which relatively few DNA sequences are compared (it is not suitable for forming a DNA databank). Because it relies on a large, intact DNA template sequence, it has some limitations in the use of degraded DNA samples. Its resolving power is much lower than targeted, species specific DNA comparison methods, such as short tandem repeats. In recent years, RAPD has been used to characterize, and trace, the phylogeny of diverse plant and animal species.

Molecular techniques, in particular the applications of molecular markers, have been used to scrutinize DNA sequence variation(s) in and among the crop species and create new sources of genetic variation by introducing new and favourable traits from landraces and related crop species.

Markers can aid selection for target alleles that are not easily assayed in individual plants, minimize linkage drag around the target gene, and reduce the number of

generations required to recover a very high percentage of the recurrent parent genetic background. Improvements in marker detection systems and in the techniques used to identify markers linked to useful traits, has enabled great advances to be made in recent years.

Though restriction fragments length polymorphism (RFLP) markers have been the basis for most of the work in crop plants, valuable markers have been generated from random amplification polymorphic DNA (RAPD) and amplified fragments length polymorphism (AFLP). Simple sequence repeats (SSR) or microsatellite markers have been developed more recently for major crop plants and this marker system is predicted to lead to even more rapid advances in both marker development and implementation in breeding programs.

Identification of the markers linked to useful traits has been based on complete linkage maps and bulked segregant analysis. However, alternative methods, such as the construction of partial maps and combination of pedigree and marker information, have also proved useful in identifying marker/trait associations. A revision of current breeding methods by utilizing molecular markers in breeding programs is, therefore, crucial in the present scenario.

References

1. Kumar, A. Molecular Markers.
http://www.science20.com/humboldt_fellow_and_science/molecular_markers
2. KeyGene Quantar Suite Versatile marker scoring software
3. Zabeau, M and P. Vos. 1993. Selective restriction fragment amplification: a general method for DNA fingerprinting. European Patent Office, publication 0 534 858 A1, bulletin 93/13.
4. Vos P, Hogers R, Bleeker M, et al. (November 1995). "AFLP: a new technique for DNA fingerprinting". Nucleic Acids Res. 23 (21): 4407–14. PMID 7501463. PMC 307397. http://nar.oxfordjournals.org/cgi/pmidlookup?view=long&pmid=7501463.