BIOCHEMICAL AND MOLECULAR-BASED SEED TESTING METHODS

Presented by Prof. Eunice Mutitu

at the Seed Enterprise Management Institute (SEMIS) short course held on 26th Sept. 2012

INTRODUCTION

- Seed health according to the International Seed Testing Association (ISTA) refers to the presence or absence of disease causing organisms such as fungi, bacteria and viruses and animal pests such as nematodes, insects and physiological conditions such as trace element deficiency.
- The commonly used methods for diagnosis include the blotter method and the agar plate method. In the blotter method, a working sample of four hundred seeds is used. These methods are good for fungi with sporulating fungi like rice blast, various blights caused by fungi like *Fusarium*, *Alternaria* etc.
- When dealing with bacteria, various biochemical tests are done in addition to determine the seedborne infections such as *Pseudomonas, Xanthomonas* and *Agrobacterium*.

SEMIs UoN
Seed Enterprises Management Institute
University of Nairobi

Cont’d

- Such tests include;
- -Gram stain test
- -Potassium hydroxide solubility test
- -Arginine dehydrolase test among many others
- Other tests are protein based or molecular based.

SEMIs UoN
Seed Enterprises Management Institute
University of Nairobi

PROTEIN BASED TESTS

- The basis of protein based tests is the formation of antibodies in the body in response to antigens. Antigens are foreign substances that elicit immune responses. The antibodies belong to a special group of serum proteins called immunoglobulins. These immunoglobulins belong to 5 classes IgG, IgA, IgM, IgD and IgE. IgG is the most prevalent.
- If an antigen is mixed with an antibody a visible precipitate occurs. The general shape of the antibodies is Y-Shaped and the antigen has binding sites at the two tips of the Y arms. This precipitation forms the basis of immuno assays that include microtitre plate methods, ELISA and agglutination tests. Serology is based on these reactions

SEMIS UoN
Seed Enterprises Management Institute
University of Nairobi

Cont’d

- Enzyme Linked Immunosorbent Assay – ELISA
- The principle of the ELISA technique follows a number of steps as shown below:
- A specific antibody is adsorbed on a plate and the excess is washed
- The test sample containing the virus (antigen) is added and the excess washed off
- The enzyme-labeled specific antibody is added followed by another wash
- The appropriate enzyme is added to the substrate
- A colour characteristic of a successful reaction develops and the scores are made depending on the colour intensity
- Many virus diseases and even some bacteria like *Ralstonia* on potatoes are detected this way.

SEMIs UoN
Seed Enterprises Management Institute
University of Nairobi

MOLECULAR BASED TESTING METHODS

- Methods of testing seed health are many and varied.
- Conventional ones are;
  - -Visual
  - -Microscopy
  - -Biochemical eg carbon utilization
- Immunochemical –protein based;
  - - ELISA
  - - Gel electrophoresis
- Molecular based ;
  - - PCR
  - -Gene sequencing
  - -RFLP

SEMIs UoN
Seed Enterprises Management Institute
University of Nairobi

Cont'd

- Why Molecular methods
  - To confirm and complement the conventional ones
  - To refine the results by use of stable hereditary characters
  - To provide alternative ways of detection when conventional ones are not adequate
- What is involved;
  - Analysis of nucleic acids – Deoxyribonucleic acid (DNA) The process involves;

- Extraction of DNA – many methods are available eg
  - CTAB method
  - use of kits e.g. Qiagen kits
  - FTA method
- Determination of concentration and quality by (i) Spectrophotometer (ii) Gel electrophoresis
- Use of restriction enzymes or endonucleases followed by gel electrophoresis
- Use of hybridization – Southern blotting
- Use of polymerase chain reaction(PCR)

SEMIs UoN
Seed Enterprises Management Institute
University of Nairobi

WHAT IS DNA

- DNA is a molecule that encodes genetic information
- It is a long coiled double stranded chain of interlocking basepairs called double helix
- It consists of subunits called nucleotides made up of a phosphate group, a pentose sugar(deoxyribose) one of four organic base molecules
- The bases are; Purines - adenine and guanine
  Pyrimidines - cytosine and thymine
- The bases pair with one another with hydrogen bonds such that adenine pairs with thymine and cytosine with guanine

SEMIs UoN
Seed Enterprises Management Institute
University of Nairobi

POLYMERASE CHAIN REACTION

- **PCR** - Makes many copies of DNA fragments using a thermocycler
- Made possible by the consequent/inherent behavior of DNA when subjected to various temperatures;

  -At 92-94oC DNA denatures – Separation of the DNA strands. The hydrogen bonds are broken

  -At 55oC the DNA anneals, strands establish the hydrogen bonds again

  -At 72oC the initiated DNA strand extends with the help of a special DNA polymerase
- **PCR REQUIREMENTS**

  -The DNA strand that needs to be copied, dNTPs – deoxynucleotides (dTTP, dATP, dCTP, dGTP)

  -Taq polymerase – Thermostable enzyme

  -Primers – Oligonucleotides that start the replication process

  -A thermocycler to run many cycles like 30 cycles

SEMIs UoN
Seed Enterprises Management Institute
University of Nairobi

ANALYSING PCR PRODUCTS

- By gel electrophoresis followed by staining with Ethidium bromide
- By hybridization and radiolabelling
- By gene sequencing
- Depending on primers used can determine what pathogen you are looking for.

SEMIs UoN
Seed Enterprises Management Institute
University of Nairobi

USES OF PCR

- Used to identify specific genetic loci for diagnostic purposes
- Used to generate DNA finger prints to determine genetic relationships
- For rapid DNA sequencing
- For rapid isolation of specific sequences for further analysis

SEMIs UoN
Seed Enterprises Management Institute
University of Nairobi

DNA EXTRACTION

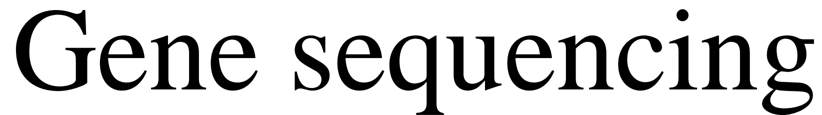
- Source if DNA can either be plants, fungi or bacteria.
- For plants tissues can either be used fresh or dried within 24 hours to allow storage at room temperature.
- Fresh material can also be frozen in liquid nitrogen and stored at minus 80oC. Ground tissue powder can also be stored at minus 80oC
- Fungal material can be harvested directly from a petridish or from liquid culture by filtration or centrifugation.
- It can the be used fresh, frozen or freeze dried

SEMIS UoN
Seed Enterprises Management Institute
University of Nairobi

Cont'd

- Material used for DNA extraction needs to be first mechanically disrupted. This is done by crushing tissue in liquid nitrogen with a glass rod or use of plasic pestle with lysis buffer. The RNA needs to be eliminated from the sample by use if RNAse enzymes and proteins and polysaccharides precipitated. The cell debris and precipitates are then removed. A binding buffer and ethanol are added to bind the DNA on a silica gel based membrane. The DNA is the eluted in small volumes of low salt buffer or water.
- For bacteria lysozyme and proteinase K with lysis buffer are used for disruption and protein removal.
- Protein removal can also be accomplished by use of phenol chloroform mixture and DNA recovered by ethanol precipitation.
- Mini kits are now available for DNA extraction where all the reagents and buffers are supplied with the kits and step by step guidance provided.
- An example is DNeasy minikit by QIAGEN.

SEMIS UoN
Seed Enterprises Management Institute
University of Nairobi



Gene sequencing

- DNA sequence involves the determination of the nucleotide sequences in DNA fragments
- It is used to reveal the fine structure of genes and other DNAs
- It confirms DNA sequence of cDNAs
- Determines the DNA sequence of promoter and othe regulatoryDNA elements that ckntrol expression

SEMIs UoN
Seed Enterprises Management Institute
University of Nairobi

Contd

- After temination electrophoresis is done on a denaturing polyacrylamide gel
- Detection is by autoradiography using x ray film
- The base sequence is read beginning from the bottom of the gel

SEMIs UoN
Seed Enterprises Management Institute
University of Nairobi

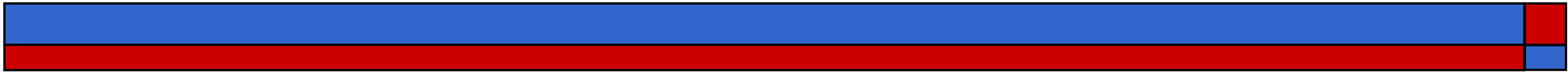
Methods of sequencing

- Sanger method also known as chain termination method
- The method is carried out like a PCR but a dideoxyribonucleotide (ddNTP) for termination is added such that ddATP is added tube1, ddTTP to tube2, ddGTP to tube3 and ddCTP to tube 4
- Termination occurs at site A,T,GandC in respective tubes

SEMIs UoN
Seed Enterprises Management Institute
University of Nairobi

- With automation the reactions are made to occur in one tube with a fluorescent dye and a laser beam used for to scan the lanes
- The different wavelengths emitted by the different nucleotides gives different colours
- The method is thus referred to as chemiluminescent DNA sequencing

SEMIS UoN
Seed Enterprises Management Institute
University of Nairobi



SEMIs UoN
Seed Enterprises Management Institute
University of Nairobi

Conclusions

- Biochemical and molecular methods complement conventional ones in seed testing
- Specialised equipment and well trained personnel are needed to carry out the tests
- Are best used to resolve or confirm cases that are not easily done conventionally

SEMIS UoN
Seed Enterprises Management Institute
University of Nairobi

SEMIs UoN

Seed Enterprises Management Institute

University of Nairobi