Practical Manual

Principals of Plant Pathology

PPA 505 3(2+1)

M.Sc. (Ag.) Plant Pathology

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2024

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Syllabus: Principals of Plant Pathology - PPA 505 3(2+1)

Practical: Basic plant pathological techniques: Isolation, inoculation and purification of plant pathogens and proving Koch's Postulates. Techniques to study variability in different plant pathogens. Purification of enzymes, toxins and their bioassay. Estimation of growth regulators, phenols, phytoalexins in resistant and susceptible plants.

Name of Student .....

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Session .....

Semester .....

Course Name: .....

Course No.: .....

Credit .....

Published: 2024

No. of copies:…..

Price: Rs.

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Date: Course Teacher

CONTENTS

Practical No. 1

Objective: Preparation of media for pathogen isolation

Activity: Prepare one litre Potato Dextrose Agar (PDA) medium. Note the materials required and quantity of components.

Materials Required:

Procedure:

Precautionary Measures:

Practical No. 2

Objective: Isolation of plant pathogen from plant tissue

Activity: Collection of infected plant samples from field and inoculation of infected sample on media. Isolate the pathogen from the given sample and identify it and note the different morphological features observed under the microscope.

Materials Required: ……

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Procedure:……

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Precautionary Measures: ……

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Practical No. 3

Objective: To study and perform Koch's Postulates

Activity: Raising healthy plants in pots and inoculation of pathogen on healthy plants

Materials Required: ……

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Procedure:……

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Observation:……

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Precautionary Measures:

Practical No. 4

Objective: Techniques to study variability in different plant pathogens

Activity: Preparation of pure culture of plant pathogens and morphological observation. Collect cultures of various pathogens from the Plant Pathology lab and observe and record morphological features of cultures and record various cultural parameters

Materials Required: ……

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Procedure: ……

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Observation:……

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Precautionary Measures: ……

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Practical No. 5

Objective: Molecular methods to assess variability using RAPD marker

Activity: Primer designing, DNA extraction and gel electrophoresis. Extract DNA from Fusarium spp., Prepare PCR mix, setup thermal conditions and perform variability study through RAPD marker

Materials Required: ……

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Procedure:……

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Observation:……

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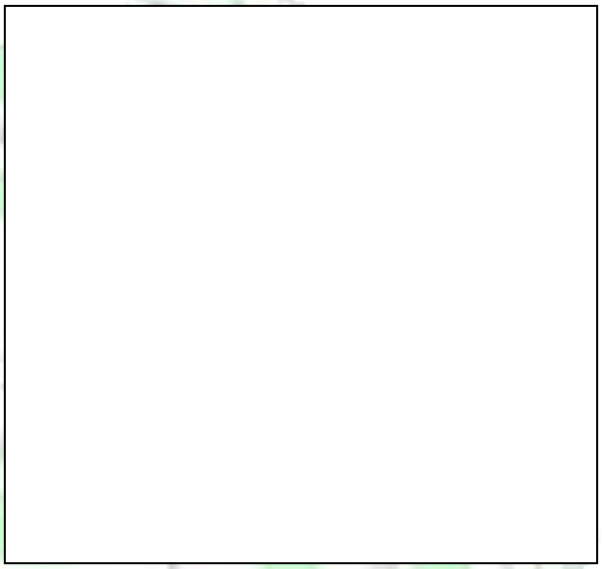

Precautionary Measures:

Practical No. 6

Objective: Molecular methods to assess variability using RFLP marker

Activity: Primer designing, DNA extraction and gel electrophoresis. Extract DNA from *Fusarium* spp., Prepare PCR mix, setup thermal conditions and perform variability study through RFLP marker

Materials Required: ……

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Procedure:……

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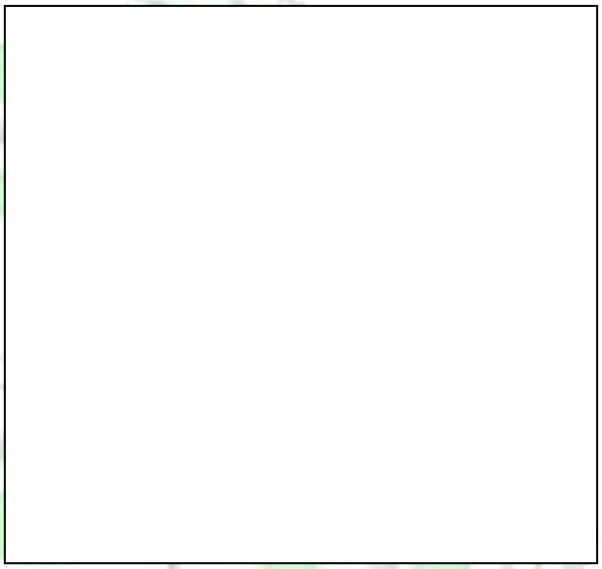

Precautionary Measures: ……

Practical No. 7

Objective: Molecular methods to assess variability using AFLP marker

Activity: Primer designing, DNA extraction and gel electrophoresis. Extract DNA from *Fusarium* spp., Prepare PCR mix, setup thermal conditions and perform variability study through AFLP marker

Materials Required: ……

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Procedure:……

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Observation:……

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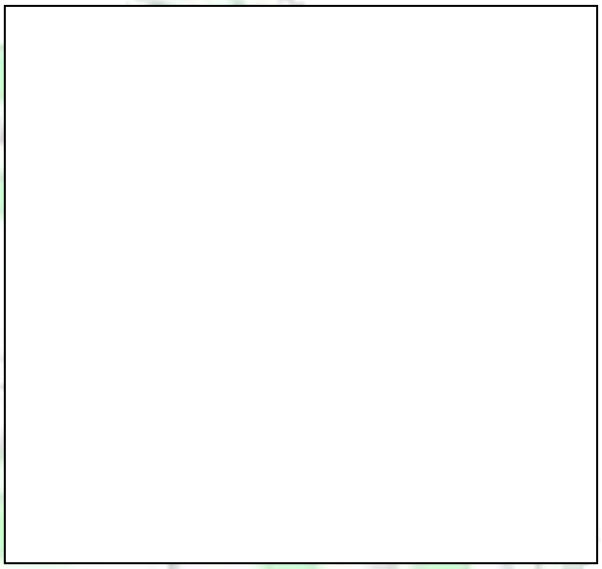

Precautionary Measures:

Practical No. 8

Objective: Molecular methods to assess variability using SSR marker

Activity: Primer designing, DNA extraction and gel electrophoresis. Assess the variability of *Fusarium moniliforme* isolated from Kanpur.

Materials Required: ……………………………………………………………………………………

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Procedure:……………………………………………………………………………………………

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Precautionary Measures: .....

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Practical No. 9

Objective: Purification of enzyme

Activity: SDS-gel electrophoresis and Gel filtration chromatography

Materials Required: ……

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Procedure:……

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Precautionary Measures: .....

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Practical No. 10

Objective: Purification of toxins

Activity: Culture of virulent strain on media, preparation of culture filtrate and isolation of toxin.

Materials Required: ……

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Procedure:……

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Observation:……

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Precautionary Measures: ……

Practical No. 11

Objective: Extraction of Phenylalanine Ammonia Lyase.

Activity: Preparation of enzyme extract and observe the OD on a spectrophotometer. Express the reaction rate as micromole trans-cinnamic acid formed per mg protein per min.

Materials Required: ……

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Procedure:……

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Calculation:……

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Precautionary Measures: ……

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Practical No. 12

Objective: Extraction of Peroxidase.

Activity: Preparation of enzyme extract and observe the OD on a spectrophotometer.

Materials Required: ……

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Procedure:……

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Calculation:……

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Precautionary Measures: ……

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Practical No. 13

Objective: Extraction of Polyphenol Oxidase

Activity: Preparation of enzyme extract and observe the OD on a spectrophotometer.

Materials Required: ……

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Procedure:……

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Calculation:……

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Precautionary Measures: ……

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Practical No. 14

Objective: Estimation of Phytoalexin

Activity: Preparation of elicitor and observe the OD on a spectrophotometer.

Materials Required: ……

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Procedure:……

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Calculation:……

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Precautionary Measures: ……

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Practical No. 15

Objective: Estimation of phenols

Activity: Preparation of enzyme extract and observe the OD on a spectrophotometer.

Materials Required: ……

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Procedure:……

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Calculation:……

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Precautionary Measures: ……

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APPENDICES

PREPARATION OF POTATO DEXTROSE AGAR (PDA) MEDIUM

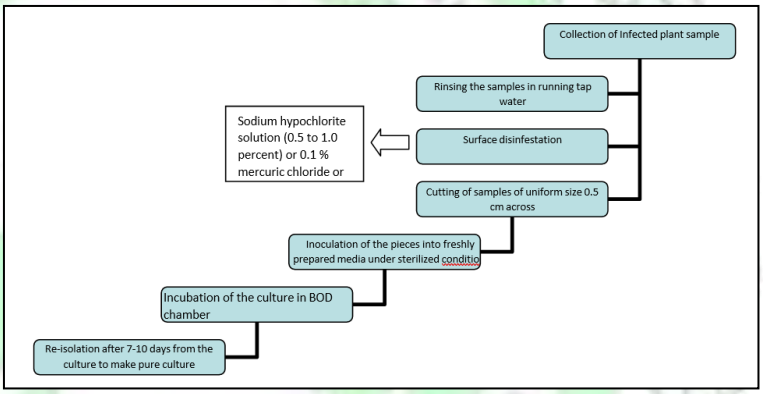
Materials required: Peeled potato – 200g; Dextrose – 20g; Agar-agar – 20g and distilled water - 1000ml, conical flask, measuring cylinder, muslin cloths, non-absorbant cotton, and ingredients in different quantities to be used as per needs

Method:

- Chopped peeled potatoes are cooked in 500 ml of water.
- Then filtered with the help of muslin cloth.
- Agar-agar is melted in 500ml of water.
- Potato juice is added to the melted agar.
- Volume is made 1000ml by adding the required water.
- Filtered again through muslin cloth.
- Dextrose is added to this mixture and shaken well.
- Medium is sterilized in an autoclave at 1.1kg/cm2 pressure for 20 minutes at a temperature of 121.6°C. Thus, the medium is ready for use.

ISOLATION OF A PLANT PATHOGEN FROM PLANT TISSUE

Tissues sampled during the active stage of infection are likely to have within them only the pathogen responsible for the infection; the surfaces of such tissues, however, are usually contaminated with saprophytic organisms. The steps for isolation of the pathogen have been given in the flowchart.



KOCH'S POSTULATES

Robert Koch was the first to show in 1876 that anthrax a disease of sheep and other animals, including humans, was caused by a bacterium that he called as *Bacillus anthracis*. Based on the studies related to anthrax, he set out the four steps that must be satisfied before a micro-organism isolated from a diseased human, animal or plant can be considered as the cause of the disease.

Four steps of Koch Postulates:

1. The suspected causal agent must be present in every diseased organism examined.
2. The suspected causal agent must be isolated from the diseased host organism and grown in pure culture.
3. When a pure culture of the suspected causal agent is inoculated into a healthy susceptible host, the host must reproduce the specific disease.
4. The same causal organism must be recovered again from the experimentally inoculated and infected host i.e., the recovered agent must have the same characteristics as the organism in step 2.

TECHNIQUES OF VARIABILITY IN PLANT PATHOGENS

The conventional methods for identifying the variability in the pathogens at species, subspecies, and intra-sub-species levels are being done by the study of virulence reactions using disease rating scales on a set of host differentials. The variability of the pathogen culture can be assessed by cultural and morphological methods. Molecular techniques are more precise tools for differentiating species and identifying new strains/ isolates. Biotechnological methods can be used to characterize pathogen populations and assess the genetic variability much more accurately.

Cultural and morphological variability

1. Culture the isolates individually on a PDA plate.
2. Cut a 5 mm disc by cork borer from the edge of the actively developing culture plates positioned at the centre of fresh PDA plates and incubate in the dark at 27 ± 1˚C.
3. After 7–8 days, observations of culture phenotypes will be recorded considering colony diameter colour, margins, and general appearance of all the Petri dishes.
4. For micro-morphological characterization observe fungal cells under a microscope.
5. Study conidial morphology. Measure length, width, and number of septations per conidia and capture microphotographs

MOLECULAR METHODS TO ASSESS VARIABILITY USING RAPD MARKER

Random Amplified Polymorphic DNA (RAPD): This is one of the simplest PCR-based molecular methods available for the characterization of pathogen populations. It uses random primers (Williams et al., 1990) and can be applied to any species without requiring any information about the nucleotide sequence. The amplification products from this analysis exhibit polymorphism and thus can be used as genetic markers. The presence of a RAPD band, however, does not allow a distinction between hetero- and homozygous states. Genetic variability is assessed by employing a short single primer of arbitrary nucleotide sequences. Specific sequence information of the organism under investigation is not required and amplification of genomic DNA is initiated at target sites which are distributed throughout the genome.

Procedure

1. Fungal DNA was extracted by using the CTAB method described by Doyle and Doyle (1990).
2. The quality and quality of the extracted fungal genomic DNA was checked in 1% agarose gel electrophoresis.
3. Take 70 ml of 0.5 X TAE buffer (10ml 50 X TAE, 990ml distilled water) and add 0.7 g of agarose and subjected to heat in a microwave oven until a clear, transparent solution is obtained.
4. After cooling for about 5 min, 2 µL of ethidiumbromide (EtBr) will be added from 10 mg/ml stock solution (0.2 g EtBr in 20 ml ddH2O) in the melted gel.
5. The melted agarose will be poured into a flatbed gel tray and a comb inserted.
6. The gel will be allowed to solidify completely at room temperature.
7. Then comb will be carefully removed and gel tray will be placed in the electrophoresis tank containing
8. 0.5 × TAE buffer.
9. DNA samples and the DNA standard marker will be loaded into the wells of the solidified gel submerged in 0.5 × TAE buffer
10. Gel electrophoresis will be carried out at 100 volts for about 40 min
11. The DNA bands in the gel were visualized using UV transilluminatior and photographed by using gel documentation system
12. Each of the five fungal DNA extracts was amplified with three different decamer primers.
13. **Conditions of RAPD-PCR:** Polymerase chain reaction (PCR) tubes containing the reaction mixture will be placed in the PCR machine. The initial process of denaturation was done at 94°C for 5 min followed by 40 cycles of denaturation at 94°C for 1 min, annealing will be done at 40°C for 1 min and final extension for 10 min at 72°C. Termination of reaction will be done at 22°C. Until further analysis on agarose gel the amplified products will be stored at 4°C.
14. The RAPD sample (25µL) will be mixed with 3µL of loading dye and the mixture will then be loaded in the wells of 1% agarose gel.
15. Electrophoresis will be carried out same as described earlier for genomic DNA. Bands will be visualized through the documentation system

MOLECULAR METHODS TO ASSESS VARIABILITY USING RFLP MARKER

RFLP procedure:

1. For PCR assays, DNA will be extracted from 1 to 6-week-old mycelium of a fungal pathogen
2. The 5.8S rRNA gene and the two flanking internal transcribed spacers (ITS1 and ITS2) will be amplified with primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS 4 (5´-TCC TCC GCT TAT TGA TAT GC-3')

3. PCR will be undertaken in 20 µl volume consisting of 5 mM of each dNTP, 20 pmol each of ITS1 and ITS4 primers, 0.5 U of Taq DNA polymerase and 100 ng of template DNA.
4. Amplifications will be done with the following cycling parameters; 94°C for 5 min, 35 cycles of 94°C for 2 min, 53°C for 2 min, 72°C for 2 min, and a final extension of 30 min at 72°C.
5. Following amplification, 5 µl of PCR products will be digested with the restriction enzymes HaeIII, EcoRI and TagI
6. The digested fragments will be separated on 1.2% agarose gel in TAE buffer, stained with ethidium bromide and visualized under UV transillumination
7. The sizes of the digested products will be determined by comparison with standard 1 kb or 100 bp molecular markers

MOLECULAR METHODS TO ASSESS VARIABILITY USING AFLP MARKER

AFLP procedure

1. The AFLP analyses will be performed with DNA from the monospore cultures
2. The DNA was restriction digested with the endonucleases EcoRI and MseI.
3. After digestion, adaptors will be ligated to the resulting fragments.
4. The fragments will then be pre-amplified using primers E (5'-GACTGCGTACCAATTC-3') and M (5'-GACGATGAGTCCTGGTAA-3').
5. Following this pre-amplification, selective PCR will be performed in which the selective primers will be nearly identical to primer E or M but will be extended by specific two- or three-nucleotide combinations at their 3' terminus.
6. Around six primer combinations will be used.
7. Primer labeling will be performed by phosphorylating the 5' end of the Eco RI primers with [γ -32P] ATP and T4 polynucleotide kinase and the amplified materials will be analysed on 5% polyacrylamide slab gels.
8. A 50-bp DNA ladder (Invitrogen) will be used as a reference.

MOLECULAR METHODS TO ASSESS VARIABILITY USING SSR MARKER

Basic PCR Protocol for a single sample

1. Prepare an ice bucket with crushed ice,
2. Bring DNAs, Primers, and PCR reagents (except for Taq DNA polymerase, see tips) as below from the stocked place (freezer), and place on ice,
3. Place PCR tubes on ice,
4. Set up a 10µL PCR reaction (Keep all your reagents on ice): 7.15 µL of DW (autoclaved distilled water) 1 µl of 10x Taq buffer; 0.8µl of dNTPs; 0.25 µL of Forward Primer (25 µM stock); 0.25 µL of Reverse Primer (25 µM stock); 0.5 µL of Template DNA (~20 ng/µL) and 0.05 µL of Taq DNA Polymerase (bring just before use from freezer)
5. Place reaction tubes in PCR thermal cycler and start program (see below).
   Prepare the pre-mixture for multiple samples:
   1. Multiply the volume of each reagent by the number of individual PCR reactions you wish to examine and add ~10% extra to account for pipetting error. In this example, you make 10 different PCR reactions (you have 10 samples to be examined), so we multiply each volume by 11 (=10+1).
   2. In a single Eppendorf tube (1.5mL) combine the following: DW: 7.15µL x 11 samples = 78.65µL
   3. 10x Taq buffer: 1µL x 11 samples = 11µL
   4. dNTPs: 0.8µL x 11 samples = 8.8µL Taq DNA polymerase: 0.05µl x 11 samples = 0.55µL (bring just before use from freezer)
   5. Mix the above contents and keep a tube on ice.
   6. Transfer 9.5µL of pre-mixture into each PCR tube.
   7. Add 0.5µL of template DNA into each sample tube.
   8. Set the tubes on the PCR thermal cycler, and start a program (see below) PCR Program
      Step1: 94°C for 3 min (Initial denaturation)
      Step2: 94°C for 30 sec (Denaturation)

Step3: 55°C for 30 sec (Annealing)
Step4: 72°C for 1 min (Extension)
Step5: repeat Step2 to 4 for 34 times (=total 35 cycles)
Step6: 72°C for 10 min (Final extension)
Step7: 4°C forever (Storage temperature)

List of materials for PCR

- PCR tubes （0.2mL or 0.5mL, up to the product specifications of the heating block in your PCR thermal Cycler,
- Ice Bucket and crushed ice,
- Extracted DNA (for PCR template),
- PCR reagent kit including 10x Taq buffer dNTPs
  Taq DNA Polymerase
- Forward Primer
- Reverse Primer
- DW (sterilized distilled water by autoclave)
- PCR Machine (thermal cycler)

PURIFICATION OF ENZYME

Objective: Maximum possible yield + maximum catalytic activity + maximum possible purity

Requirement: Enzyme sample, Standard enzyme sample, Glass beaker, Ammonium sulphate, B-mercaptoethanol, Centrifuge, Electrophoresis chamber, SDS-PAGE gel, Isoelectric focusing gel, Glass chamber, Glass slide, Silica slurry, Column washing buffer, Elution buffer, Dialysis bag, PBS Buffer

Salting In and Out:

1. Take crude enzyme solution in a beaker
2. Add ammonium sulphate to it slowly, until precipitation occurs
3. Centrifuge the mixture and collect precipitate.

Gel electrophoresis:

1. Prepare SDS-gel for electrophoresis,
2. Add β-mercaptoethanol to sample
3. Add enzyme sample in gel well along with standard,
4. Run the sample in presence of an electric field
5. Stain the gel for 10-20 min, and use destaining solution to remove excess amount of stain.
6. Observe the bands.

Iso Electric Focusing:

1. Prepare Isoelectric focusing gel by using the mould
2. Transfer the gel onto the electrophoretic chamber carefully,
3. Place a strip of paper on the side of the gel for sample application,
4. Apply the electrical parameters for separation of the sample contains,
5. Stain the gel with suitable dye for 30 to 60 min with gentle shaking,
6. Destain the gel for 30 min with gentle shaking,
7. Observe the bands against a clear background.

Gel Filtration Chromatography:

1. Prepare a column by using an agarose or agarose beads,
2. Mount the column in a vertical position by using a stand,
3. Pour filtration buffer to equilibrate the column,
4. Add a sample to top of the column,

5. Add a buffer on top of the column,
6. Connect vacuum pump to column and start it,
7. Collect elute fractions of, and repeat the process from step 5 to collect different size sample elute.

Ion-exchange Chromatography:

1. Prepare column for chromatography and mount it vertical position.
2. Pour a buffer into column to equilibrate the column and drain the buffer from bottom,
3. Ensure the column resin is settled down,
4. Load sample on it along with wash buffer 2-4 times,
5. Drain the excess buffer and add elution buffer,
6. Collect elute samples for further use.

Adsorption Chromatography:

1. Take a clean glass jar, add solvent to it,
2. Cover the jar with a lid for 30 min to equilibrate the environment,
3. Take a clean glass slide pour silica gel slurry onto it, make a uniform layer, allow it to dry,
4. Apply sample spot on the glass slide,
5. Place glass slide in the glass jar,
6. Remove the glass slide when the mobile phase rises to ¼ of the slide,
7. Stain it with a suitable stain and observe it.

Affinity chromatography:

1. Prepare the column by using the resins and allow resin to settle down,
2. Add a sample on it along with the affinity binding buffer 2-3 times,
3. Collect buffer fraction, add elution buffer,
4. Collect elute and add high-concentration salt buffer to elute the remaining binding molecules,
5. Collect 2nd elute in another tube.

Dialysis:

1. Cut the proper length of the dialysis bag,
2. Open up the bag by soaking in the dialysis buffer,
3. Tie the one end of the dialysis bag with the help of the clamp,
4. Add the sample along with the PBS buffer,
5. Kept dialysis bag in the beaker containing PBS buffer overnight, change the outer buffer every 2-3 hr.
6. Centrifuge the dialysis bag sample,
7. Collect the supernatant and pellet, and store them in cold for further use.

PURIFICATION OF TOXINS

Fungal cultures and toxin production:

1. Cultures of a virulent isolate grown on potato sucrose agar (PSA) plates
2. For toxin production, transfer four 6-cm-diameter mycelial plugs from PSA cultures into a 500-ml flask containing 200ml of potato sucrose broth (PSB)
3. Incubate the cultures at 25°C on a rotary shaker (150 rpm) under continuous diffused light for 21 days.
4. Culture filtrates are obtained by passing the liquid through four layers of cheesecloth and Whatman no. 1 filter paper.

Leaf necrosis assay.

1. The biological activities of crude and processed extracts determined by leaf necrosis assay on susceptible plant leaves.
2. For each treatment, detach 10 leaves at the three-leaf stage, create a light wound at the abaxial surface, and a 30-µl solution applied to the wound site.
3. Incubate leaves in a moist chamber at 25°C for 3 days, and the lesion area around each wound was assessed

Solvent extraction.

1. Extract culture filtrate (50 ml) three times with half volumes of petroleum ether, cyclohexane, carbon tetrachloride, ether, benzene, ethyl acetate, or chloroform using a separatory funnel.
2. Evaporate both water and solvent fractions to dryness at 50°C
3. Dissolve the residues in 50 ml of sterile distilled water, and their toxicity was measured using leaf necrosis assay.

Isolation of phytotoxin from culture filtrates.

1. After methanol precipitation, filtrate from a 21-day-old culture was extracted three times with half-volume ethyl acetate, and evaporated to dryness at 50°C
2. A yellow-brown residue was obtained and dissolved in 10 ml of hot methanol for analysis by thin-layer chromatography (TLC).
3. Coat preparative analytical TLC plates with a GF-254 fluorescent silica gel (5 × 20 cm, Qingdao, China) and spot with samples developed separately in solvent systems containing (i) normal butanol/acetone/water (6:1:3, vol/vol/vol), (ii) chloroform/methanol/water (6:3:1, vol/vol/vol), (iii) chloroform/water/formic acid (6:3:1, vol/vol/vol), (iv) normal butanol/acetic acid/water (8:2:1, vol/vol/vol), (v) normal butanol/cyclohexane/methanol (15:5:1, vol/vol/vol), (vi) ethyl acetate/petroleum ether (2:1, vol/vol), (vii) ethyl acetate/hexane (8:1, vol/vol), (viii) normal butanol/hexane/methanol (15:5:1, vol/vol/vol), or (ix) ethyl acetate/petroleum ether/methanol (4:1:0.35, vol/vol/vol)
4. Mark TLC plates using last solvent mentioned above under ultraviolet (UV) light at 254nm, and each band was then carefully scraped off the plate.
5. Dissolve scrapings in ethyl acetate, filtered through Whatman no. 1 filter paper, and centrifuged at 8,000 rpm for 10 min
6. Dry the ethyl acetate filtrate with a rotary evaporator at 50°C.
7. Dissolve a portion of each residue from separate TLC bands to 5% (wt/vol) in sterile distilled water and subjected to leaf necrosis assay
8. Several fractions showed toxicity in the leaf assay, but the one showing the highest toxicity was to be subsequently used for purification.

Purification of phytotoxin

1. Select the biologically active compound from the most active fraction on TLC plates
2. Dissolve in methanol, filtered, and then subjected to analytical high-performance liquid chromatography (HPLC) and preparative liquid chromatography (LC).
3. Collect subfractions under a 215- nm detector.
4. These subfractions were tested by leaf necrosis assay, and a bioactive compound designated as SS- toxin was further purified by LC and chosen for further analysis.

EXTRACTION OF PHENYLALANINE AMMONIA LYASE

(L-Phenylalanine Ammonia Lyase EC 4.3.1.5). Phenylalanine ammonia-lyase (PAL) is responsible for the conversion of L-Phenylalanine to trans-cinnamic acid. Cinnamic acid serves as a precursor for the biosynthesis of coumarins, isoflavanoids, and lignin. These compounds play an important role in pest and disease resistance mechanisms. Changes in PAL activity accompanying fungal, viral, and bacterial infection of plants have been reported.

Principle: Phenylalanine Ammonia lyase activity is determined spectrophotometrically by following the formation of trans-cinnamic acid which exhibits an increase in absorbance at 290 nm (crude enzyme)/270nm (purified enzyme).

Materials: Borate Buffer 0.2 M (PH 8.7), L-Phenylalanine 0.1 M, 1M Trichloro Acetic Acid. Dissolve 16.3g in 100 ml water.

Enzyme Extract: Homogenize 500 mg of the plant material in 5 ml of cold 25 mm borate-HCL buffer pH 8.8 containing 5mm mercaptoethanol (0.4 ML/L). Centrifuge the homogenate at 12,000g for 20 min. Use the supernatant as an enzyme source.

Procedure

1. Pipette out 0.5 ml borate buffer, 0.2 ml enzyme solution, and 1.3 ml water in the test tube.
2. Initiate the reaction by the addition of 1 ml L-Phenylalanine solution.
3. Incubate for 30-60 min at 32°C.
4. Stop the reaction by the addition of 0.5 ml of 1m trichloroacetic acid.
5. Run a control in which add phenylalanine after trichloroacetic acid.
6. Measure the absorbance at 290 nm.
7. Prepare a standard graph for trans-cinnamic acid.

EXTRACTION OF PEROXIDASE

(Donor; H_2O_2 Oxidoreductase E.C. 1.11.1.7). Peroxidase (POD) includes in its widest sense a group of specific enzymes such as NAD- Peroxidase, NADP-Peroxidase, fatty acid peroxidase etc. As well as a group of very non-specific enzymes from different sources which are simply known as POD (donor; H_2O_2-Oxido reductase 1.11.1.7). POD catalyzes the dehydrogenation of a large number of organic compounds such as phenols, aromatic amines, and hydroquinone sets. POD occurs in animals, higher plants and other organisms. The best studied is horse radish POD.

Principle: Guaiacol is used as a substrate for the assay of peroxidase. Guaiacol+H_2O_2 $\rightarrow$ POD Oxidized guiacol+2H_2O. The resulting oxidized (dehydrogenated) guaiacol is probably more than one compound and depends on the reaction conditions. The rate of formation of guaiacol dehydrogenation product is a measure of the POD activity and can be assayed spectrophotometrically at 436nm.

Materials

- Phosphate Buffer 0.1 M (pH 7.0).
- Guaiacol Solution 20 mm.
- Dissolve 240 mg guaiacol in water and make to 100 ml. It can be stored frozen for many months.
- Hydrogen peroxide solution (0.042% = 12.3 mm). Dilute 0.14 ml of 30% H_2O_2 to 100ml with water. The extinction of this solution should be 0.485 at 240 nm. Prepare freshly.

Enzyme Extract: Extract 1 g of fresh plant tissue in 3 ml of 0.1 M phosphate buffer pH 7 by grinding in a pre-cooled mortar and pestle. Centrifuge the homogenate at 18,000 g at 5°C for 15 min. Use the supernatant as an enzyme source within 2-4 hours. Store on ice till the assay is carried out.

Procedure

1. Pipette out 3 ml buffer solution, 0.05 ml guaiacol solution, 0.1 ml enzyme extract, and 0.03 ml hydrogen peroxide solution in a cuvette (Bring the buffer solution to 25°C before assay).
2. Mix well. Place the cuvette in the spectrophotometer.
3. Wait until the absorbance has increased by 0.05. Start a stopwatch and note the time required in minutes (Δt) to increase the absorbance by 0.1.

Calculation: Since the extinction coefficient of guaiacol dehydrogenation product at 436 nm under the conditions specified in 6.39 per micromole, the enzyme activity per litre of extract is calculated below:

$$\text{Enzyme activity units/litre} = \frac{3.18\times0.1\times1000}{6.39\times1\times\Delta t\times0.1} = \frac{500}{\Delta t}$$

Notes:

1. Most accurate values are obtained when Δt is between 1 and 3 min. The enzyme extract has therefore to be diluted appropriately.
2. O-dianisidine (1 mg/ml methanol) may be used as an alternative substrate for the assay. The oxidized O-dianisidine (yellow/orange colored compound) is measured at 430 nm. Take 3.5 ml phosphate buffer (pH 6.5) in a clean dry cuvette. Add 0.2 ml enzyme extract and 0.1 ml freshly prepared O-dianisidine solution. Bring the assay mixture to 28-30°C and then place the cuvette in the spectrophotometer set at 430 nm. Then, add 0.2 ml 0.2 M H2O2 and mix. Immediately start the stopwatch. Read the initial absorbance and then at every 30-sec interval up to 3 min. If the rate of increase is very high, repeat the assay with diluted extracts. Plot

increase in absorbance against time. From the linear phase, read the change in absorbance per minute. Express enzyme activity in terms of rate of increased absorbance per unit time per mg protein or tissue weight. A water blank is used in the assay.

EXTRACTION OF POLYPHENOL OXIDASE

(Monophenol, dihydroxyphenylalanine: Oxygen oxidoreductase EC 1.14.18.1)

Phenol oxidases are copper proteins of wide occurrence in nature that catalyze the aerobic oxidation of certain phenolic substrates to quinones which are autoxidized to dark brown pigments generally known as melanins. These enzymes are assumed to be single enzymes with broad specificity although there is some evidence for the presence of more than one phenol oxidase in certain tissues. Each enzyme tends to catalyze the oxidation of one particular phenol or phenolic compound more readily than others. The polyphenol oxidase (PPO) comprises of catechol oxidase and laccase. The activities of these enzymes are important in (a) plant defence mechanism against pests and diseases and (b) the appearance, palatability, and use of plant products. Fresh fruits, vegetables, mushrooms, etc. contain these enzymes considerably.

Principle: The intensely yellow 2-nitro-5-thiobenzoic acid (TNB) with an absorption maximum at 412 nm reacts with the quinones generated through enzymatic oxidation of 4-methyl catechol (catechol oxidase) and 1,4 dihydroxybenzene (laccase) to yield colourless adducts. The decrease in the absorbance of yellow colour due to enzyme activity is measured.

Materials required: Citrate-phosphate buffer 0.2 M (pH 6.0).

- **Preparation of 2-nitro-5-thiobenzoic anion (TNB):** Add 30 mg sodium borohydride to a suspension of Ellman's reagent, i.e. 5, 5-dithiobis (2-nitrobenzoic acid) (19 mg) in 10 mL water. Within 1h, the disulphide is quantitatively reduced to the intensely yellow, water-soluble thiol. This solution is stable for at least one week when stored at 4 °C.
- **Preparation of the Quinine Solutions:** Dissolve 4-methyl -1, 2- benzoquinone in double-distilled water in a 50 mL volumetric flask by bubbling nitrogen gas until the quinone is completely dissolved.
  Prepare p-benzoquinone solution also in a similar manner. Both solutions are stable for 30 min, a time sufficient to carry out the spectrophotometric assay.
- **Substrate Solution:** 4-methyl catechol (2 nM) for catechol oxidase assay Quinol (1,4dihydroxybenzene, 2 mM) for laccase assay
- **Enzyme Extract:** Prepare the first acetone powder of fresh plant tissue (see under indole acetic acid oxidase). To get a crude enzyme preparation, mix 100 mg acetone powder with 2.5 mL of 0.2 M citrate phosphate buffer (pH 6.0), and 1 mL of 1% Triton X-100. 6.5 mL of water and 500 mg polyamide. Shake for 1 h and filter enzyme source.

Procedure

1. Pipette out into a clean 1 cm cuvette of 1.4 ml citrate 0.1 M phosphate buffer (pH 6.0), 0.5 ml of TNB and 1 ml of the substrate solution.
2. The reaction is initiated by the addition of 0.1 ml of enzyme preparation and immediately note down the absorbance at 412 nm in a spectrophotometer already set.
3. Follow the decrease in absorbance at 30-second intervals and record.

ESTIMATION OF PHYTOALEXIN

Phytoalexins are antimicrobial substances synthesized in plants upon pathogen infection. Phytoalexin is also a valuable indicator of plant defence response. Phytoalexin can be quantitated in various methods from classical organic chemistry to Mass-spectrometry analysis. This procedure has high reproducibility and simplicity that can easily handle large numbers of treatments. The method only requires a spectrophotometer as laboratory equipment, and does not require any special analytical instruments (e.g., HPLC, mass spectrometers, etc.) to measure the phytoalexin molecule quantitatively, i.e., most scientific laboratories can experiment.

Materials and Reagents: Spatula–smooth narrow tip and smooth glass rod, Plastic Petri dishes (60 x 15 mm), Plastic container with wet, Kimwipes inside for humidity, Paper towel or Kimwipe, Immature pea pods (1.5-2.0 cm in length) grown in sand and clay pots at 65-70 F under greenhouse conditions and freshly harvested (use within

3 h of applying a treatment). Remove the calyx and retain it briefly in sterile water. Endocarp will be used for the assay, Glass vials of 30 ml, Candidate elicitor solutions best dissolved in deionized water, DMSO, Hexane, 95% ethanol.

Equipment

1. Adjustable pipettes (P-200 and P-1000 and corresponding tips)
2. Flask 500 ml with 5 ml dispenser top or 5 ml pipet for dispensing hexane
3. Glass beakers, 30 ml
4. Room temperature dark cabinet space for pathogen or elicitor treatments (as described in step 3b)
5. UV spectrometer
6. 1 cm Path length quartz cuvettes

Procedure

1. **Preparation of elicitors:** The selection of elicitors is by design open to innovation. Follow the directions of manufacturers for solubility procedures. Water-soluble compounds dissolved at near-neutral pH are preferred. When solubility depends on ethanol, DMSO etc., there must be suitable control applications with only the respective solvent. Incompatible pathogens can be a positive control for an inducer of non-host resistance. See Note 2 in detail.
2. **Preparation of pod halves:**
    a. Harvest pods and remove calyx. Hold these pods in a sterile deionized water reservoir to keep the tissue moist.
    b. Select uniform-sized and conditioned pea pods from their water reservoir.
    c. Separate the pod halves with a smooth spatula avoiding wounding as much as possible.
    d. Fresh weight of pod halves is determined.
    e. Lay the endocarp (inner) surface layer up in a Petri dish
3. **Application of elicitors**
    a. Apply 25 µl of elicitor candidate solution and lightly distribute over the entire surface with a glass rod. For the control, apply the same solvent used for dissolving the elicitor.
    b. Treated pods are retained in a plastic container with wet paper towels) to maintain humidity and then incubate in the dark or moderate light for up to 24 hours.
4. **Extraction and measurement of Phytoalexin (pisatin)**
    a. Pods are transferred to 30 ml glass vials using forceps and immersed in 5 ml of hexane for 4 h in the dark. Typically, 400 mg fresh weight per 5 ml of hexane.
    b. The hexane is decanted off into 30 ml beakers and the hexane is evaporated in the air stream of a hood in low light because pisatin is not stable in bright light (typically light strength in the lab is not incandescent).
    c. One milliliter of 95% ethanol is added to the residue and read at 309 nm in a cuvette using a spectrophotometer.
    d. To ensure purity, a UV spectrum is measured in the range of 220-320 nm to verify the characteristic spectrum.

Data analysis: After subtracting the OD309 value of the non-treated control, the numbers are converted based on the equation: 1.0 OD309 unit = 43.8 µg/ml pisatin in 1 cm path length (see note 4). Data should be shown with pisatin quantity (µg) per fresh weight of tissues (g). Data obtained should be analyzed using ANOVA followed by Student's t-test. The difference with $P < 0.05$ is considered significant.

Notes

1. The pea endocarp tissue has some potential to condition or partially take up small percentages of insoluble materials in suspension, such as the cell wall fragments released by fungal spores, detected in an electron microscope view (Hadwiger et al., 1981).
2. The effect of elicitors can be compared with that of an incompatible fungal pathogen, e.g., *Fusarium solani* f. sp. *phaseoli* (*F.s.ph*.) is a bean pathogen (not for pea).
3. Pisatin has been purified from the final step with thin-layer chromatography and mass spectrometry (Teasdale et al., 1974; Seneviratne et al., 2015). The assay is accurate because pisatin only absorbs at 309 nm.

4. Pisatin in ethanol has a characteristic UV absorption spectrum with two peaks at 286 nm and 309 nm. When pisatin is the only light-absorbing compound in the solution, the ratio OD309 to OD286 is 1.47 (Cruickshank and Perrin, 1961).

Estimation of Phenols: Phenols, the aromatic compounds with hydroxyl groups, are widespread in plant kingdom. They occur in all parts. Phenols are said to offer resistance to diseases and pests in plants. Grains containing high amounts of polyphenols are resistant to bird attack. Phenols include an array of compounds like tannins, flavonols etc. Total phenol estimation can be carried out with the folin-Ciocalteant.

Principle: Phenols react with phosphomolybdic acid in the Folin-Ciocalteau reagent in an alkaline medium and produce a blue-coloured complex (molybdenum blue).

Materials required: 80% Ethanol, Folin-Ciocalteau Reagent, Na2CO3, 20%, Standard (100 mg Catechol in 100ml Water). Dilute 10 times for a working standard.

Procedure

1. Weigh exactly 0.5-1.0 g of the sample and grind it with a pestle and mortar in a 10-time volume of 80%ethanol.
2. Centrifuge the homogenate at 10,000 rpm for 20 min. Save the supernatant. Re-extract the residue with five times the volume of 80% ethanol, centrifuge and pool the supernatants.
3. Evaporate the supernatant to dryness.
4. Dissolve the residue in a known volume of distilled water (5 ml).
5. Pipette out different aliquots (0.2-2ml) into test tubes.
6. Make up the volume in each tube to 3 ml with water.
7. Add 0.5 ml of Folin-Ciocalteau reagent.
8. After 3 min, add 2 ml of 20% Na2CO3 solution to each tube.
9. Mix thoroughly. Place the tubes in boiling water for exactly one minute, cool, and measure the absorbance at 650 nm against a reagent blank.
10. Prepare a standard curve using different concentrations of catechol.

Calculation: From the standard curve find out the concentration of phenols in the test sample and express as mg phenols/100 g material.

Notes: If any white precipitate is observed on boiling, the colour may be developed at room temperature for 60 min. Express the results in terms of catechol or any other phenol equivalents used as standard.