Practical manual

PRINCIPLES OF PLANT PATHOLOGY

APP-505 3(2+1)

For

M.Sc. (Ag.) Plant Pathology

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Department of Plant Pathology
College of Agriculture

Rani Lakshmi Bai Central Agricultural University
Jhansi, Uttar Pradesh-284003

Practical manual on

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Content

Practical No. 1

Preparation of media for pathogen isolation

Objective: To prepare Potato Dextrose Agar medium

Materials required: For the preparation of potato dextrose agar medium the following ingredients in different quantities are used

(i) Peeled potato slices - 200g

(ii) Dextrose - 20g

(iii) Agar- agar - 20g

(iv) Distilled water - 1000 ml

Method:

(1) Potato slices are cooked in 500 ml of water.

(2) Then filtered with the help of muslin cloth.

(3) Agar-agar is melted in 500 ml of water.

(4) Potato juice is added to the melted agar.

(5) Volume is made 1000 ml by adding required water.

(6) Again lit is filtered through muslin cloth.

(7) Dextrose is added in this mixture and shaken well.

(8) Medium is sterilized in an autoclave at 1.1kg/cm^2 pressure for 20 minutes at temperature of 121.6°C. Thus the medium is ready for use.

Activity : Prepare one litre of Potato dextrose Agar medium. Note the materials required and quantity of the components.

Practical No. 2

Isolation of a plant pathogen form plant tissue

Objective: Isolation of plant pathogens from Diseased Plant Tissues

Tissues sampled during the active stage of an 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 of isolation of the pathogen have been given in the flowchart below:

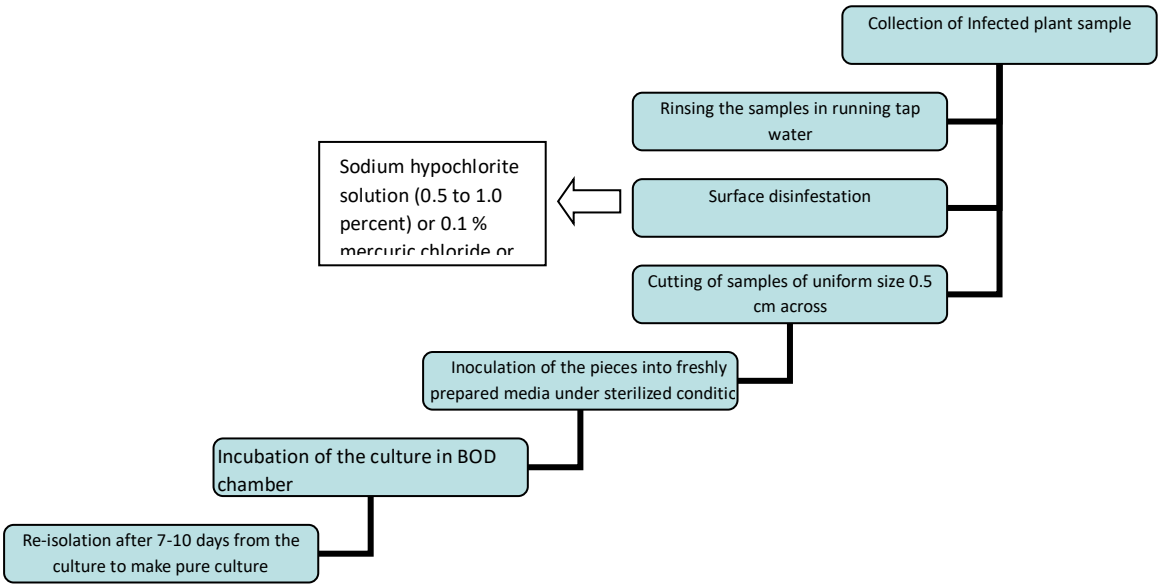

Fig. 1. Flowchart showing steps of isolation of pathogen from plant tissues

Activity: Isolate the pathogen from the given sample and identify it and note the different morphological features observed under the microscope.

Practical No. 3

To study and perform Koch Postulates

Objective: To Prove 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.

Activity: Perform all the steps of Koch Postulates.

Practical No. 4

Techniques to study variability in different plant pathogens.

The conventional methods for identifying the variability in the pathogens at species, subspecies, and intra-sub-species level is being done by the study of virulence reactions using disease rating scales on a set of host differentials. Variability of the pathogen culture can be assessed by cultural and morphological methods. Molecular techniques are more precise tools for differentiation between species, and identification of 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 PDA plate.
2. Cut a 5 mm disc by cork borer from the edge of the actively developing cultureplates and positioned at the centre of fresh PDA plates andincubated 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 from 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

Activity: Collect cultures of various cultures of pathogens from Plant Pathology lab and observe and record morphological features of cultures and record various cultural parameters

Practical No. 5

Molecular methods to assess variability using RAPD marker

1. RAPD
2. RFLP
3. AFLP
4. SSR
5. ISSR
6. rDNA markers

1. **Random Amplified Polymorphic DNA (RAPD)** This is one of the simplest PCR based molecular methods available for the characterization of pathogen population. 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 distinction between hetero- and homozygous states. Genetic variability is assessed by employing short single 123 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 target fungal genomic DNA was isolated by doing 1% agarose gel electrophoresis.
3. To 70 ml of 0.5 × TAE buffer(10ml 50 × TAE, 990ml distilled water) 0.7 g of agarose will be added and subjected to heat in a microwave oven until a clear, transparent solution 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 20ml ddH2O) in the melted gel.
5. The melted agarose will be poured into a flat bed gel tray and comb inserted.
6. The gel will be allowed to solidify completely at room temperature.
7. Then comb will carefully removed and gel tray will be placed in the electrophoresis tank containing 0.5 × TAE buffer.
8. DNA samples and the DNA standard marker will be loaded into the wells of the solidified gel submerged in 0.5 × TAE buffer
9. Gel electrophoresis will be carried out at 100 volts for about 40 min
10. The DNA bands in the gel were visualized using UV transilluminatior and photographed by using gel documentation system

11. Each of the five fungal DNA extract was amplified with three different decamer primers. In order to determine genetic variability among different isolates of target fungi
12. 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.
13. RAPD sample (25 µL) will be mixed with 3 µL of loading dye and the mixture will then loaded in the wells of 1% agarose gel.
14. Electrophoresis will becarried out same as described earlier for genomic DNA. Bands will be visualized through the documentation system

    Activity: Extract DNA of *Fusarium* spp and perform variability study through RAPD marker

Practical No. 6

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 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 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

Activity: Extract DNA of *Fusarium*spp and perform variability study through RFLP marker

Practical No. 7

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 *Eco*RI and *Mse*I.
3. After digestion, adaptors will be ligated to the resulting fragments.
4. The fragments will then pre-amplified using primers E (5'-GACTGCGTACCAATTC-3') and M (5'-GACGATGAGTCCTGGTAA-3').
5. Following this preamplification, selective PCR will be performed in which the selective primers will 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 -^{32}P] 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.

Activity: Extract DNA of*Fusarium*spp and perform variability study through AFLP marker

Practical No. 8

Molecular methods to assess variability using SSR marker

Basic PCR Protocol for single sample

1. Prepare ice bucket with crashed ice

2. Bring DNAs, Primers, 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 10 µL 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)

    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

    10x Taq buffer: 1µL x 11 samples = 11µL

    dNTPs: 0.8µL x 11 samples = 8.8µL Taq DNA polymerase: 0.05µl x 11 samples = 0.55µL (bring just before use from freezer)

    3. Mix the above contents and keep tube on ice.

    4. Transfer 9.5µL of pre-mixture into each PCR tube.

    5. Add 0.5µL of template DNA into each sample tube.

    6. Set the tubes on the PCR thermal cycler, and start 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 crashed 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)

Activity: Assess the variability of *Fusariummoniliforme* isolated from four districts of Bundelkhand

Practical No.9

Purification of enzyme

Objectives : maximum possible yield + maximum catalytic activity + maximum possible purity

Requirement:

1.	Enzyme sample
2	Standard enzyme sample
3	Glass beaker
4	Ammonium sulphate
5	B-mercaptoethanol
6	Centrifuge
7	Electrophoresis chamber
8	SDS-PAGE gel
9	Isoelectric focusing gel
10	Glass chamber
11	Glass slide
12	Silica slurry
13	Column washing buffer
14	Elution buffer
15	Dialysis bag
16	PBS Buffer

Salting In and Out:

1. Take crude enzyme solution in a beaker

2. Add ammonium sulphate to it slowly, until precipitation occur
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 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 mold
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 vertical position by using a stand,
3. Pour filtration buffer to equilibrate the column,
4. Add sample to top of the column,
5. Add buffer on top of the column,
6. Connect vacuum pump to column and start it,
7. Collect elute fraction's 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 in column to equilibrate the column and drain the buffer frombottom,
3. Ensure the column resin is settle 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 sample for further use.

Adsorption Chromatography:

1. Take a clean glass jar, add solvent to it,

2. Cover the jar with lid for 30 min to equilibrate the environment,
3. Take a clean glass slide pour silica gel slurry on to it, make 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 mobile phase uprises up to ¼ of the slide,
7. Stain it with suitable stain observes it.

Affinity chromatography:

1. Prepare the column by using the resins and allow resin to settle it down,
2. Add sample on it along with the affinity binding buffer for 2-3 times,
3. Collect buffer fraction, add elution buffer,
4. Collect elute and add high concentration salt buffer to elute 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 sample along with the PBS buffer,
5. Kept dialysis bag in the beaker containing PBS buffer for overnight, change the outer buffer every 2-3 hr,
6. Centrifuge the dialysis bag sample,
7. Collect the supernatant and pellet, and store in cold for further use.

Practical No. 10

Purification of toxins

Objective: The objectives of this study were to purify the major phytotoxin related to a virulent pathogen, and to assess biological activities of this phytotoxin.

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 200 ml 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 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 light wound at 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 extracted three times with half-volume ethyl acetate, and evaporated to dryness at 50°C
2. A yellow-brown residue obtained and dissolved in 10 ml of hot methanol for analysis by thin-layer chromatograph (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 the last solvent mentioned above under ultraviolet (UV) light at 254 nm, and each band was then carefully scraped off the plate.
5. Dissolve scrapings in ethylacetate, 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 highest toxicity 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 tested by leaf necrosis assay, and a bioactive compound designated as SS-toxin was further purified by LC and chosen for further analysis.

Practical No. 11

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)/270 nm (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 5 mm mercaptoethanol (0.4 ML/L). Centrifuge the homogenate at 12,000g for 20 min. Use the supernatant as enzyme source.

Procedure

1. Pipette out 0.5ml borate buffer, 0.2 ml enzyme solution, and 1.3ml 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℃.
4. Stop the reaction by the addition of 0.5 ml of 1m trichloroacetic acid.
5. Run a control in which add phenylalanine after trichoroacetic acid.
6. Measure the absorbance at 290 nm.
7. Prepare a standard graph for trans-cinnamic acid.

Calculation

Express the reaction rate as micromole trans-cinnamic acid formed per mg protein per min.

Practical No.12

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-Oxidoreduxtase 1.11.1.7).POD catalyzes the dehydrogenated of a large number of organic compounds such as phenols, aromatic amines, hydroquinonesets. 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 $\underline{\text{P}}$ODOxidized 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 makeup 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℃ for 15 min. Use the supernatant as enzymesource within 2-4 h. 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℃ before assay).
2. Mix well. Place the cuvette in the spectrophotometer.
3. Wait until the absorbance has increased by 0.05. Start a stop-watch and note 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 is 6.39 per micromole, the enzyme activity per litre of extract is calculated as below:

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

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 coloured 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 H_2O_2 and mix. Immediately start the stopwatch. Read the initial absorbance and then at every 30 sec intervals 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 min. 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.

Practical No.13

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 enzyme with broad specificity although there is some evidence for the presence of more than one phenol oxidase in certain tissues. Each individual 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 enzymes are important with regard to (a) plant defense mechanism against pests and diseases band (b) appearance, palatability, and use of plant products. Fresh fruits, vegetables, mushroom, 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-methylcatechol (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-methylcatechol (2 nM) for catechol oxidase assay Quinol (1,4dihydroxybenzene, 2 mM) for laccase assay
- **Enzyme Extract**

Prepare first acetone powder of fresh plant tissue (see under indole acetic acid oxidase). To get a crude enzyme preparation, mix 100 mg acetone powder wth 2.5 mL of 0.2 M citrate phosphate buffer (pH 6.0) , 1 mL of 1% Triton X-100. 6.5 mL of water and 500 mg polyamide. Shake for 1 h and filteras enzyme source.

Procedure

1. Pipette out into clean 1 cm cuvette of 1.4 ml citrate 0.1 M phosphate buffer (ph 6.0), 0.5 ml of TNB and 1ml 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 sec intervals and record.

Practical No. 14

Estimation of Phytoalexin

Phytoalexins are antimicrobial substance synthesized in plants upon pathogen infection. Phytoalexin is also a valuable indicator of plant defense response. Phytoalexin can be quantitated in various methods from classical organic chemistry to Mass-spectrometry analysis. This procedure is with high reproducibility and simplicity that can easily handle large numbers of treatments. The method only requires a spectrophotometer as laboratory equipment, 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 perform the experiment.

Materials and Reagents

1. Spatula–smooth narrow tip and smooth glass rod
2. Plastic Petri dishes (60 x 15 mm)
3. Plastic container with wet Kimwipes inside for humidity
4. Paper towel or Kimwipe
5. 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 calyx and retain briefly in sterile water (Figure 1). Endocarp will be used for the assay (see Note 1 in detail)
6. Glass vials 30 ml
7. Candidate elicitor solutions best dissolved in deionized water (For exceptions see Procedure 1)
8. DMSO
9. Hexane
10. 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 Pathlength quartz cuvettes

Procedure

1. **Preparation of elicitors**

The selection of elicitors is by design open to innovation. Follow the directions of manufactures 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 pathogen can be a positive control for an inducer of nonhost 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 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 h.

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 for4 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 evaporated in the air streamof a hood in low light because pisatin is not stable in bright light (typically light strength inthe lab is not incandescent).

c. One milliliter of 95% ethanol is added to the residue and read at 309 nm in a cuvette usingspectrophotometer.

d. To insure purity, a UV spectrum is measured in the range of 220-320 nm to verify thecharacteristicpisatin spectrum.

Data analysis

After subtracting the OD_{309} value of non-treated control, the numbers are converted based on the equation: 1.0 OD_{309} unit = 43.8 µg/ml pisatin in 1 cm pathlength (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. 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 electron microscope view (Hadwiger et al., 1981).

2. Effect of elicitors can be compared with that of an incompatible fungal pathogen, e.g., Fusariumsolanif.sp. phaseoli (Fsph), which is a pathogen for bean (not for pea). An example result was shown in Figure 3, in which the data show that no detectable pisatin above background in watertreated tissue, and accumulation of pisatin induced by a fungal wall carbohydrate (i.e., chitosan) and by an authentic inducer of nonhost resistance in pea, a fungal pathogen of bean (i.e., Fsph).

3. Pisatin has been purified from the final step with both 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).

Practical No. 15

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 amount 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 Folin-Ciocalteau reagent in alkaline medium and produce blue coloured complex (molybdenum blue).

Materials required

- 80% Ethanol
- Folin-Ciocalteau Reagent
- Na_2CO_3, 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 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% Na_2CO_3solution to each tube.
9. Mix thoroughly. Place the tubes in boiling water for exactly one min, 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:

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