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SDS-PAGE of Unknown Pr Protein Reveals 1, 4 Glucanase after Disease Development in Edible Cowpea (*Vigna unguiculata*) sp.

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Abstract: *This annual herbaceous legume is an excellent crop for farmers due to its tolerance for sandy soil and low rainfall. It requires very few inputs, as the plants root nodules are able to fix atmospheric nitrogen, well-suited to intercropping with other crops. The whole plant is used as forage for animals, with its use as cattle feed likely responsible for its name.*

The crop is selected to identify the pathogenic microorganism causing the disease in the selected plant (Vignaunguiculata sesquipedalis), isolate the obtained pathogenic forms in their pure culture, determine the effect of interaction between the obtained pathogen, identify the PR (pathogenesis related) protein being produced by the infected fruit of the plant which is induced by the pathogenic microorganism. After isolation of the microorganism slide bioassay was done using fungicides Blitox and Bavastine to observe the change in growth and germination pattern of the pathogen. The molecular basis of plant–pathogen interactions remains an intensely active area of investigation. Stress related genes extensively used in biotechnology have been cited in this paper. Stress related proteins identified must be followed through for studying the molecular mechanism for plant defense against pathogens.

Keywords:*pathogen related protein, plant-pathogen interactions, plant defense*

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I. Introduction

Vignaunguiculata subsp. *sesquipedalis* is a legume cultivated to be eaten as green pods. It is known as the yardlong bean, bora, bodi, long-podded cowpea, asparagus bean, pea bean, snake bean, or Chinese long bean. Despite the common name, the pods are actually only about half a yard long; the subspecies name sesquipedalis (one-and-a-half-foot-long) is a rather accurate approximation of the pods' length. This plant is of a different genus from the common bean. It is a vigorous climbing annual vine. The plant is subtropical/tropical and most widely grown in the warmer parts of South Asia, Southeast Asia, and southern China.

Genus:*Vigna*
Species: *unguiculata*
Subspecies: sesquipedalis

The crop is selected to conduct the following experiments:

- To identify the pathogenic microorganism causing the disease in the selected plant (*Vignaunguiculata sesquipedalis*).
- To isolate the obtained pathogenic forms in their pure culture.
- To determine the effect of interaction between the obtained pathogen if any.
- To identify the PR (pathogenesis related) protein being produced by the infectedfruit of the plant which is induced by the pathogenic microorganism.

The molecular basis of plant–pathogen interactions remains an intensely active area of investigation. Stress related genes extensively used in biotechnology have been cited in this paper. Stress related proteinsidentified must be followed through for studying the molecular mechanism for plant defense against pathogens.

II. Materials And Methods

2.1 Isolation and Staining

2.1.1. Materials required

Infected leaves of *V.unguiculatasesquipedalis* was obtained from the fields	Blade
Sterile water	Potato(for sectioning)
Mercuric chloride solution (0.1%)	Forceps
PDA slants	Needle
NA slants	Gram's staining reagent
Lactophenol cotton blue stain	

2.1.2. Method

Infected leaves with specific lesions were selected and washed in sterile water to remove mud and dust and cut into pieces. These pieces were surface sterilized with 0.1% mercuric chloride ($HgCl_2$) solution and transferred aseptically to **PotatoDextroseAgar (PDA)** slant containing streptomycin and Nutrient Agar slant for isolating fungi and bacteria and incubated for isolating pure colonies. The pure colonies were chosen and marked as Sample B and sample F for bacterial and fungal culture respectively.
Sample B was stained by gram staining and sample F was stained by Lactophenol cotton blue staining in two different slides and observed under a microscope.

2.2. Koch's postulate

2.2.1. Materials required

Fresh uninfected fruit of *V. unguiculata* (borboti)	Fungal suspension
Forceps	Syringe
Sterile water	Cellotape

2.2.2. Method

To confirm the pathogenicity of both bacteria and fungi by Koch postulate, healthy fruits of same borboti plant were inoculated with suspension of Sample B and F and incubated at room temperature

1. Firstly fungal spores' suspension of Sample F obtained from the infected plant leaves was made. 0.1ml of inoculum was then inoculated into a fresh fruit of *V. unguiculata* sand inoculated areas were covered with cello tape. 0.1ml of sterile water was injected into another fruit and kept as the control.
2. *The fruits were kept in moist cotton bed and kept at room temperature for 2-3 days.*

2.3. Slide bioassay

2.3.1. Materials required

Slides	Sterile water
Cotton	Sugar solution (2%)
Alcohol	Fungicides blitox and Bavastine (100ppm each)
Microtips	Cover slips
Fungal suspension	Bacterial suspension

2.3.2. Method

In this investigation the slide bioassay was done. Fungicides Blitox and Bavastine were used to observe the change in growth and germination pattern of the pathogen. Two sets of slides were used in which two were control slides.
1. Firstly eight slides were taken and were made grease free.
2. Then only spore suspension and Distilled water was taken in a dropwise manner in the first slide at one side of the slide and its replica taken on the other end of the slide. In this way replica was made at one side of the original in all the eight slides. Then suspension with water was covered by a cover slip and this slide was marked as Control slide. (Control 1)
3. In the second slide fungal spore and sugar suspension were taken in the similar manner like the first one and this slide was marked as the positive control (control 2)
4. In the third slide spore suspension and Fungicide 1 was taken in the similar way, in the fourth slide spore suspension sugar solution and Fungicide 1 were taken along with their replicas at one side of slide.
5. In the fifth slide Spore suspension and Fungicide 2 and in the sixth slide spore suspension, sugar solution and Fungicide 2 were taken with their replicas in the similar way.

6.In the 7th slide spore suspension (Of sample F) and sample B were taken and in the 8th slide spore suspension,Actinomyces and sugar solution were taken with their replicas on one side in the similar way.

2.4. Protein estimation

2.4.1. Materials required

Infected and Control seed	Cheesecloth	Eppendorf tubes
Mortar and pestle	Funnel	Standard BSA Solution (2mg/ml)
Tris Buffer	Falcon tubes	Bradford's Reagent
Distilled water		

2.4.2. Preparation of 2mg/ml stock BSA: The stock of 20mg/ml solution (not more than 10ml in the 15ml centrifuge) was done. We took1ml of stock and madeup the volume to 10ml. Now this is 2mg/ml working solution.

Bradford Reagent (5X concentrate)

100 mg -Coomassie Brilliant Blue G-250
47 ml - Methanol (100%)
100 ml -Phosphoric Acid (85%)
QS to 200 ml with H_2O

2.4.3. Method

Protein estimation was done after extracting the proteins from both the infected and the control fruit. The protein was extracted by mechanical agitation (by crushing the infected seed and the control seed separately with mortar and pestle in buffer) followed by filtration and centrifugation. After centrifugation the supernatant was taken. The standard curve for protein estimation was done by Bradford's method. The OD (at 595nm) of the protein sample from the infected and control fruit were plotted in the Y axis against concentration of BSA in the X axis in order to determine the protein content in both the samples.

2.5. SDS –PAGE

SDS-PAGE was done to separate the protein samples obtained from the infected and the control fruit. Three lanes were loaded (marker lane, lane containing infected protein and lane containing control protein). The following were the steps.

III. Results

Table 1. Physical Characterization of leaf.

Characteristics	**Normal Leaf**	**Infected Leaf**
Location of sample collection	Field	Field
Description: Leaf shape Leaf Surface Leaf margin	 Oval Rough Wavy	 Oval Rough Wavy
Nature of contamination	------	Fungal
Description of Lesion Shape of lesion Size of lesion Colour of lesion Pattern of lesion Texture of lesion	 ------ ------ ------ ------ ------	 Circular Spore diameter = 36.63µm Small lesions Brown with yellow tints Rough

Table 2. Chemical Characterization of leaf.

Characteristics	**Normal Leaf**	**Infected Leaf**
SPM	0.167 gm.	0.066gm
pH	4.86	6.11
Electrical Conductivity	104.6	140.3

By comparing the physical characteristics of infected and normal leaf the spore diameter of the infected leaf was found to be 36.63µm. the pH and the electrical conductivity increased while SPM decreased when compared to the normal uninfected leaf.

Sample	Probable microorganisms
F	*Cladosporium* sp.
B	*Actinomycetes*

Table 2. Result of Koch's postulate.

Name of pathogen	**Number of points inoculated in the healthy fruit**	**Number of points infected**	**Appearance and nature of spots or lesions**	**Percentage of infection**
Sample B	3	0	**NONE**	0
Sample F	3	2	Yellow powdery growth around area of inoculation Halo present around area of inoculation.	66.66

Sample B was identified as non-pathogenic while sample F (Cladosporium sp.*) was identified as pathogenic.*

Table 3. Result of slide bioassay

Name of the slide	**Suspension of microorganism**	**Solution**	**Length of germ tube**	**% of germination**
Control 1	Sample F Suspension	Sterile water	16.65	14.55
Control 2	Sample F Suspension	Sugar (2%)	33.75	20.83
Slide 1	Sample F Suspension + Sample B Suspension	Sterile water	15.34	12.58
Slide 2	Sample F Suspension +Sample B Suspension	Sugar (2%)	18.32	2.30
Slide 3	Sample F Suspension	Fungicide 1 (100ppm)	4.45	18.47
Slide 4	Sample F Suspension	Fungicide 2 (100ppm)	4.47	15.23

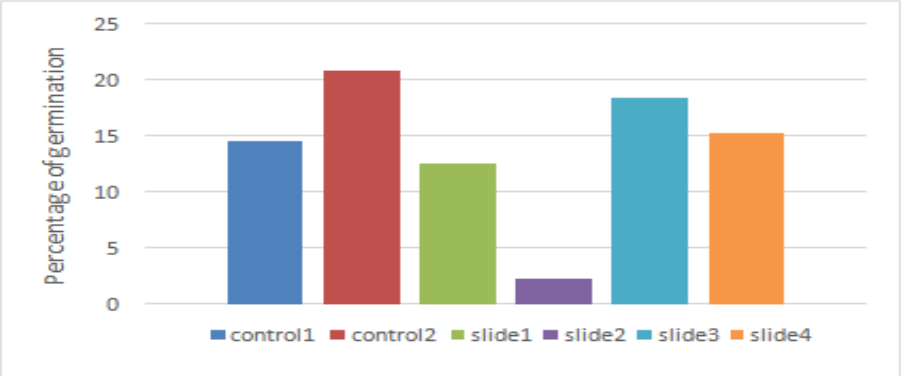

Figure 1- The graphical representation of changes in percentage of germination observed in slide bioassay**.**Y axis denotes the percentage of germination of the test culture and X axis denotes the test slides.

- There was no noticeable interaction between sample B and Sample F.
- On treatment with fungicide1 and 2 the germ tube length decreased.

Table 4. For protein estimation: Construction of standard curve by Bradford's method.

Test tube no.	**BSA standard concentration(2mg/ml)**	**Volume of BSA (ml)**	**Volume of distilled water (ml)**	**Bradford reagent(ml)**	**Incubation at Room temperature for 30 minutes**	**Absorbance at 595nnm**
1	0	0	2	5 ml		0
2	0.4	0.4	1.6			1.71
3	0.8	0.8	1.2			1.94
4	1.2	1.2	0.8			2.01
5	1.6	1.6	0.4			2.24
6	2.0	2	0			2.11

Infected	Unknown	1	1			1.23
Control	Unknown	1	1			0.64

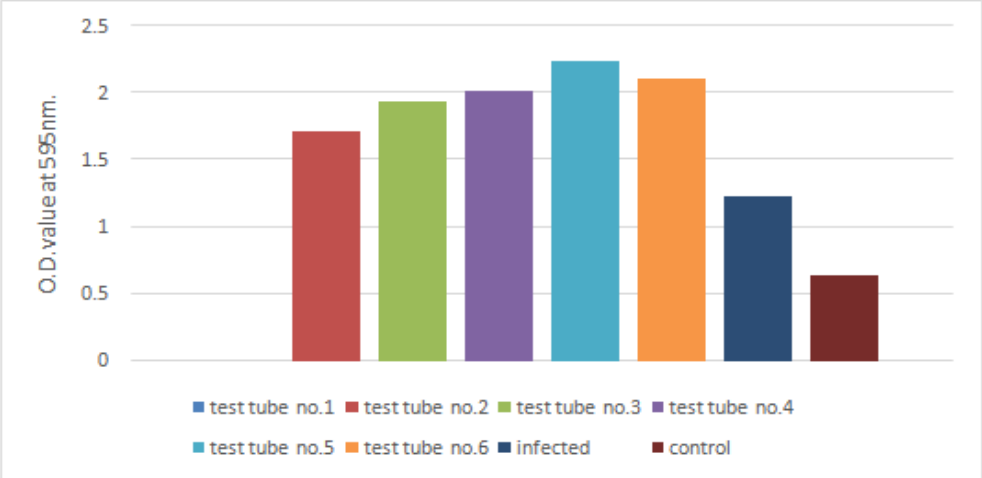

Figure 2: The graphical representation of the changes in the O.D. values in the reaction medium. Y axis denotes the O.D. values of the test culture solutions at 595nm and X axis denotes the test isolates.

Result of SDS-PAGE

By comparing the protein bands (obtained after performing SDS PAGE) between the protein ladder (marker) and the infected protein sample, a 55kd protein was identified as beta 1, 4 glucanase.

IV. Conclusion and Discussion

After the experiment it was observed that the concentration of the protein in the infected sample was found to be almost double than that of the protein concentration of the control sample.

- Protein concentration of infected sample =1.23 mg/ml
- Protein concentration of the control sample = 0.64 mg/ml

Pathogenesis-related (PR) proteins are proteins produced in plants in the event of a pathogen attack. They are induced as part of systemic acquired resistance. Infections activate genes that produce PR proteins.[11] Infections also stimulate the cross-linking of molecules in the cell wall and the deposition of lignin, responses that set up a local barricade that slows spread of the pathogen to other parts of the plant. The changes in the pattern of protein synthesis in germinating maize embryos have been examined after infection with the fungus Fusarium moniliforme.[9] In vivo labelling experiments showed that infection with F. moniliforme results in the induction of the synthesis of two major acid-soluble polypeptides with mol. wts of 24 and 17 kDa and with highly basic isoelectric points. In another finding regulation of 1,3 glucanase has been shown to occur at the mRNA level in barley.[15] These beta 1,3 glucanases accumulated to high levels in a manner consistant with the observed rise in steady state levels of beta 1,3 glucanases inmRNA. This parallel accumulation of PR protein with mRNA suggests that beta 1,3 gene expression may be regulated at the level of mRNA accumulation.[10]

Fungal pathogens produce several elicitors and these elicitors serve as primary signaling molecules in triggering or switching on PR genes. Cis –acting elicitor responsive element have been identified in promoters of the class I chitinase gene CHN50 of tobacco.[5] Similarly a fungal elicitor (arachidonicacid) from Phytopthora infestans induced activation of the potato PR gene, PR -10 a. From our discussions we can say that in our selected plant *Vigna unguiculata sesquipedalis* similar analogus genes might be switched on which produces beta 1, 4 endoglucanse as a PR protein. The beta 1, 4 endoglucanse catalyses the hydrolysis of cellulose.[14]

PR genes encoding PR and stress related proteins are widely used in biotechnology. Knowledge of the full complement of proteins expressed by the genome of a cell, tissue, or organism at a specific time point (the proteome) is necessary to understand the biology of a cell or organism.[7] As the proteome reflects the actual state of the cell or the organism under any given set of conditions, it is an essential bridge between the transcriptome and the metabolome.[10]

To produce stress-tolerant cultivars, it is imperative that the genes whose products confer stress tolerance areidentified. Identification of stress-responsive genes requires a multi-disciplinary approach that includes genome-wide genetic and physical chromosome mapping, isolation and sequencing of important genes, microarray analysis, and proteomic analyses.[3]

A 55kd protein β 1, 4 glucanase was identified as a PR Protein in *Vigna unguiculata* which is induced by fungi *Cladosporium* sp. Further experiments can be performed to identify the genes that regulate this protein. This is important to produce stress tolerant cultivars of the crop.

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