# Protein analysis for study of spot blotch disease in wheat

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## Abstract

Spot blotch disease of wheat is now considered as one of the major constraints in wheat growing regions specially in South East Asia, Australia and Latin American countries where warm humid conditions persist during wheat crop season. Due to the tremendous breeding efforts, new partially resistant lines have recently been released. However, the lack of complete resistant genotypes and insufficiency of knowledge about the resistance mechanism still hamper sustained breeding efforts.Comparative investigationof the interaction of *Bipolarissorokiniana* with moderate resistant genotype Chirya 3 and susceptible genotype DDK 1025 will help in understanding the genes involved in pathogen resistance. Hence, Plant pathogen system under greenhouse conditions was established and tissue was collected after 24 hr. of inoculation. Protein was extracted from collected tissue and used for protein extraction method optimization for quantitative expression analysis using 2-D gel electrophoresis. Protein extraction protocol optimized using TCA-acetone extraction protocol was found to be best suitable for plant pathogen interaction analysis. The study of *B. sorokiniana* and wheat interaction at protein level will give overview of resistance mechanism.

**Keywords:** *Bipolaris sorokiniana, Protein, SDS-PAGE, IEF*

## Introduction

Wheat is considered as the most widely grown and consumed food crop of the world and is the staple food of around 35% of the world's population. The present (2023) wheat production is about 787 million tons(http://faostat.fao.org/) and to feed the world's ever-growing population in 2050, there will be a requirementto produceabout 1040 million tons of wheat (Pingali et. al., 1999). To reach this target, it is crucial to keep the crop free from various abiotic as well as biotic stresses.In recent years, spot blotch, caused by *Bipolarissorokiniana*, has emerged as a serious concern for cultivation of wheat in warmer and humid regions of the world. Extensive economic loss in wheat production has occurred due to the severity of spot blotch in the last 20 years (Sharma and Duveiller, 2006).Spot blotch is a major biotic constraint for wheat in the Gangetic plains and is the main restraining factor forwheat production in South-East Asia (Chowdhury, et. al., 2013). Nearly 12 million hectares of land under cultivation are affected in South East Asia and Latin American countries(Nagarajan and Kumar 1998). The pathogen affects almost all the crops belonging to *Graminacae* family (Pandey et al., 2005).

Exact yield losses due to this fungal pathogen have been reported to be difficult, due to co-infecting pathogens in the field (Mattias, 2008).However, reports show the estimated yield losses up to 15.5 to 19.6% (Dubin and Van Ginkel 1991), 20 to 80% (Duveiller and Gilchrist 1994), which may reach upto 100% under severe infection conditions (Mehta, 1998). Currently, *B. sorokiniana* is a pathogen of major concern at national level in India and its frequency is highest in north-eastern plains zone amongst the six agro climatic zones due to the prevalence of hot and humid weather conditions (Sjöberg, 2005).

*Bipolarissorokiniana* is a hemibiotrophic fungus having both biotrophic and necrotrophic phases. The biotrophic growth phase is limited to a single epidermal host cell, while invasion of the mesophyll tissue and host cell death is the characteristic of thenecrotrophicgrowth phase. *B. sorokiniana* causes foliar spot blotch, root rot, black point on grains, head blight and seedling blight of wheat and barley (Kumar et. al., 2002).

Barley and wheat are the most economically important plantsthat are infected by this fungus. The infection usually starts from seeds, infected soils or from host debristhat transmits conidia via physical contact or rain splashes. Foliar spot blotch leads to decrease the photosynthetic capacity of theleaf, resulting in early senescence. Common root rot

decreases the water andnutrition uptake efficiency, causing weak seedlings which subsequently wither and die. In avirulent *B. sorokiniana*strain, about 90% of the conidia starts togrow, forming germ tubes and germlings or hyphae after 3h only. The germ tubes and hyphae of *B. sorokiniana*are surrounded by extra cellular matrix (ECM) that provides a beneficial environment for the fungus (Åkesson et. al., 1995; Apoga and Jansson, 2000).

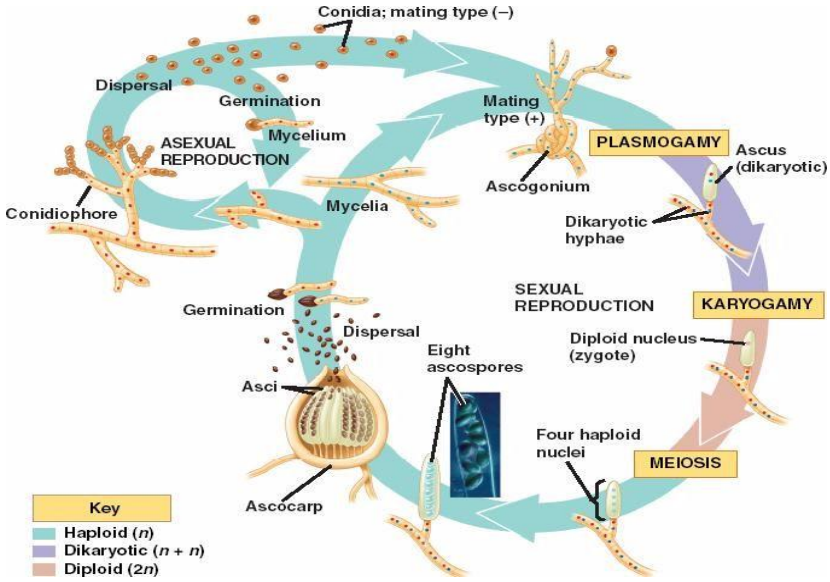



**Figure1: Life cycle of Ascomycetes (*B. sorokiniana*)**

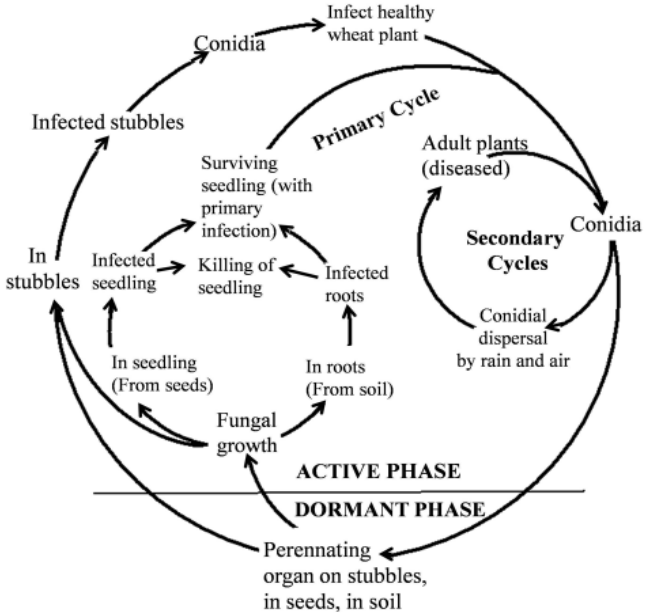



**Figure2: Disease cycle of *Bipolaris sorokiniana***

At the initial stage of infection there is no sign of chlorotic margin. In case of a susceptible genotype the small lesions extend very rapidly and ultimately reach into

several centimeters in the later stage (Jones and Clifford 1983; Mathre, 1987). Often yellowingextending from the lesion is observed due to toxin production. Later such spots coalesce each other thus result blight on large leaf portion.Disease incidents of wheat caused by *B. sorokiniana*can be controlled in a number of ways. Integrated pest management is by far the best method of controlling the pathogen (Mehta, 1993; Dubin and Duveiller 2000). Such program integrates the use of: (i) cultural practice, (ii) crop rotation, (iii) seed treatment, (iv) biological control, (v) foliar fungicide and (vi) disease resistant varieties (Acharya et. al., 2011, Kumar et. al., 2020).

Despite several studies, no effective controlling measure has been devised yet.In order to achieve any ideal controlling measure for plant disease, it is very important to understand the mechanism involved in defense response during plant-pathogen interaction.This research has focused on the details of the interaction using protein analysis approach for identification of novel resistant proteins.

## Material and Methods

### *Samples required for Spot blotch system in green house*

- DDK 1025 seeds:*Triticumdicoccum*, susceptible to spot blotch
- Chirya 3 seeds: *Triticumaestivum*, moderate resistant to spot blotch
- *Bipolarissorokiniana* isolate

### a. Plant pathogen system setup

Seeds of DDK 1025 and Chirya 3 were sterilized by giving 1% NaOCl treatment for 5 minutes followed by 3 washes of sterile distilled water, 5 minutes each.Sterilized seeds were imbibed in sterile water for 6 hrs followed by *in vitro* germination under aseptic conditions and then transferred to after germination. Germinated seeds were sown in autoclaved soil rite and allowed to grow in greenhouse.At two leaf stage, half of the plants were inoculated with spore suspension ($2x10^3$/ml) of *B. sorokiniana*while rests were mock inoculated with water having 0.1% tween-20 for control.Plants were regularly monitored for growth.

### b. Tissue collection

Leaf tissue was collected from the above experiment for protein analysis. Tissue was collected at 24 hr. post inoculation in 3 biological replicates. Collected leaf tissue

was immediately frozen in liquid Nitrogen and was stored at -80ºC. The four different samples were collected and labelled as follow

1. SI: Susceptible Inoculated plant (DDK 1025 inoculated)

2. SC: Susceptible Control plant (DDK 1025 control)

3. RI: Moderate resistant inoculated plant (Chirya 3 inoculated)

4. RC: Moderate resistant control plant (Chirya 3 control)

**c. Protein extraction**

Protein extraction was performed using the TCA- acetone precipitation method. For this purpose, 500 mg of leaf tissue was finely grind in liquid nitrogen and 1 ml of 10% TCA in acetone containing 2 % β-ME was added to each tube. Tube was kept at -20˚C for 1 hr. and spinned it at 16,000 rpm for 1 hr. and then supernatant was removed. Pellet was washed 3 times with ice cold 100% Acetone by 30 min spin at 12000 rpm at 4˚C. Supernatant was removed and the pellet was retained. Pellet was driedby evaporating the acetone. 1 ml of IEF buffer ( CHAPS 4%, Thio-Urea 2 M, Urea 8 M, DTT 18 mM )was added to it and spinned it at 16,000 rpm for 30 min. The supernatant was taken gently into another tube and stored at -80˚C.

**d. Protein Quantification**

Protein extracted using TCA-acetone precipitation method was quantified using Bradford protein. A series of Bovine Serum Albumin (BSA) standard solutions of known concentrations were prepared by diluting a 2 mg/ml stock solution of BSA in distilled water. Standards were prepared as shown in Table 1. For quantification, 50 µl of the protein from tissue samples were dilutedto make equal volume (500 µl) using distilled water. The concentration of the protein sample should be within the linear range of the standard curve. Then, add 500 µl volume of Bradford reagent to each tube and mix well by inverting the cuvette several times. The cuvettes were incubated at room temperature for 5 minutes. During this time, the Bradford reagent binds to the protein, resulting in a shift in the dye's absorption spectrum and a change in color from brown to blue.Absorbance was measured at 595 nm in Dual beam Spectrophotometer (Lab-India, India).

**Table 1:** Preparation of standards of BSA for Protein estimation

| *Tube no.* | *BSA Standard (2mg/ml) Volume (µL)* | *Milli Q water (µl)* | *Final BSA concentration* |
|---|---|---|---|
| **1** | 500 | 0 | 2 |
| **2** | 375 | 125 | 1.5 |
| **3** | 250 | 250 | 1.0 |
| **4** | 187.5 | 312.5 | 0.75 |
| **5** | 125 | 375 | 0.50 |
| **6** | 62.5 | 437.5 | 0.25 |
| **7** | 31.25 | 468.75 | 0.125 |

**e. SDS-PAGE:** SDS- PAGE was performed for extracted proteins. 10 µg of protein was loaded onto 5% stacking gel on the top of 10% resolving gel using a Bio-Rad Page system and allowed to run under applied constant voltage (80 V). Gels were prepared using the components as mentioned in the Table 2.

**Table 2:** Components of SDS-PAGE gels

| **Component** | **5% Stacking Gel** | **10% Resolving Gel** |
|---|---|---|
| 40% Acrylamide | 0.5 ml | 5.0 ml |
| 1.5 M Tris (pH 6.8) | 1.25 ml | 5 ml |
| SDS | 100 µl | 2.5 ml |
| 2% bis-Acrylamide | 0.1 ml | 2.6 ml |
| Milli Q Water | 3 ml | 4.8 ml |
| APS | 25 µl | 100 µl |
| TEMED | 5 µl | 10 µl |
| Final Volume | 5 ml | 20 ml |

**f. 2-D Polyacrylamide Gel Electrophoresis**

Two-dimensional gel electrophoresis (2-D electrophoresis) was performed for leaf tissue proteins extracted using TCA-Acetone extraction protocol. Proteins from each sample were loaded onto 7 cm gel strip having pH range 3-10 for one dimensional electrophoresis. Buffer containing 0.5% carrier ampholyte containing 100 µg of protein from each tissue sample was incubated in strips for 16 h for rehydration. The rehydrated strips were focused on Isolelectric focusing unit (Bio-Rad, India) at 50-250 V for 30 min. and 250-4000 V for 2 h, followed by 4000-10000 Vhr for a period of 2 h. Focused strips were washed with equilibration buffer I (20% Glycerol, 1.5 M Tris pH 8.8, 6 M Urea, 2% SDS, Dithiotritol 2%) for 15 min. Strips were then transferred to equilibration buffer II

(20% Glycerol, 1.5 M Tris pH 8.8, 6 M Urea, 2%SDS, Iodoacetamide 2.5%) for 15 min. These equilibrated strips were then placed on 10% resolving gel and were sealed using 0.5% agarose containing Bromophenol Blue tracking dye. After isoelectric focusing, these strips were further subjected to PAGE for second dimensional electrophoresis as per the details of the gels prepared as mentioned in Table 2.

After completion, the gel was removed and stained using silver staining protocol.Fix the proteins using fixative (40 % methanol, 10 % acetic acid, 50 % water) for overnight. Wash the gel with 30 % ethanol (wash solution) three times for 20 min each. Reduce the proteins by adding reducing solution (0.02% solution of sodium thiosulphate) and incubate for 1 min. Wash the gel with water three times for 30 sec. Add 50 ml of Silver stain (2 gm silver nitrate, 200 µl formaldehyde in 1 liter water) to the gel for 20 min (use chilled solution). Wash the gel with water three times for 30 sec. Add the Developer solution (30 gm sodium carbonate, 5 mg sodium thiosulphate, 500 µl formaldehyde in 1 liter water) until the protein bands are visible. Add the Stop solution and leave the gel in this solution.

## Results

### *Protein Quantification and SDS-PAGE*

Extracted proteins were stored in -80ºC. The protein yield using Bradford method was found to bein the range of 25-27µg/gm of leaf tissue as shown by absorbance in Table 3.

**Table 3:** Absorbance of protein samples observed at 595 nm

| S. No. | BSA conc. | 1st Set | 2nd Set | Average |
|---|---|---|---|---|
| 1 | 0.125 | 0.172 | 0.174 | 0.173 |
| 2 | 0.250 | 0.244 | 0.242 | 0.243 |
| 3 | 0.50 | 0.417 | 0.375 | 0.396 |
| 4 | 0.75 | 0.565 | 0.545 | 0.555 |
| 5 | 1.0 | 0.880 | 0.878 | 0.879 |
| 6 | 1.5 | 0.925 | 0.934 | 0.932 |
| 7 | 2 | 0.971 | 0.971 | 0.931 |
| 8 | SC | 0.523 | 0.527 | 0.525 |
| 9 | SI | 0.546 | 0.534 | 0.540 |
| 10 | RC | 0.523 | 0.529 | 0.526 |

| | | | | |
|---|---|---|---|---|
| 11 | RI | 0.516 | 0.520 | 0.518 |

Extracted protein was analysed for quality assessment before 2D-PAGE using SDS-PAGE run as shown in Figure 3.

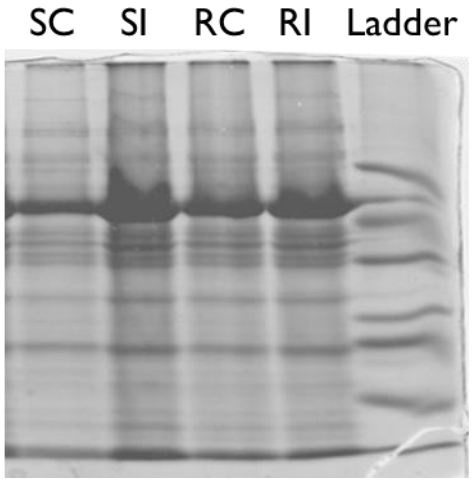
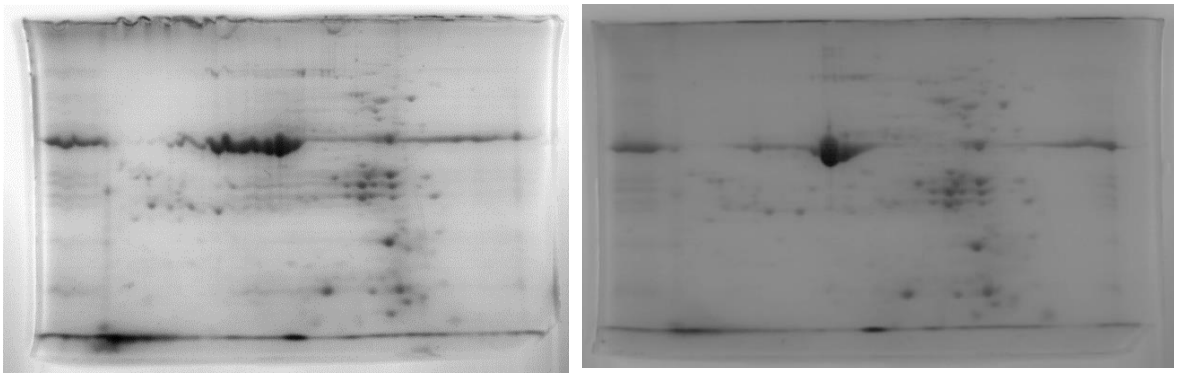


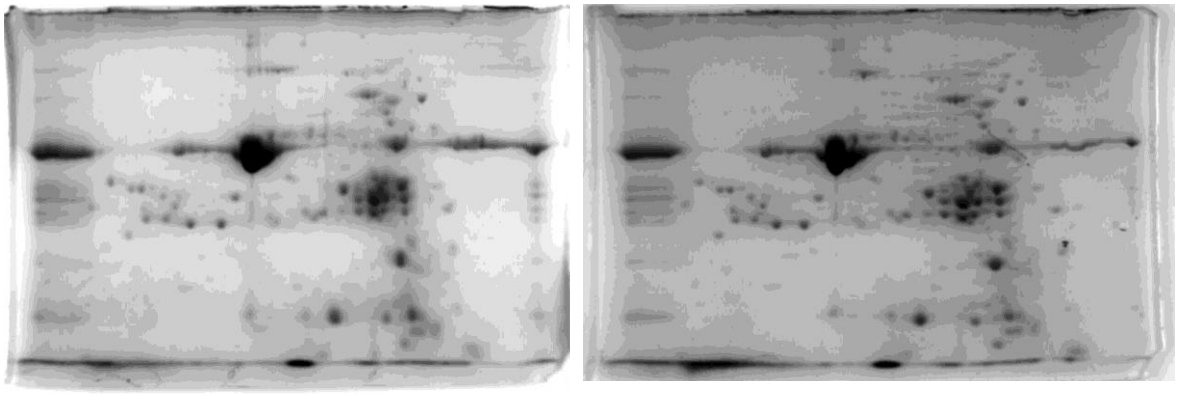

**Figure 3:**Four samples analysed for protein quality on SDS-PAGE

***2-Dimensional PAGE Analysis:***

After 2-DPolyacrylamide Gel electrophoresis,the gels were observed for differential protein expression. The gels showed difference in their band pattern for several proteins (Figure 4).



(A) (B)



(C) (D)

**Figure 4:** 2D-PAGE gel images of protein extracted from spot blotch leaf samples (A) SI (B) SC (C) RI (D) RC

**Discussion**

In wheat, little is known aboutthe physiological and molecular events regulating geneexpression under spot blotch conditions. It is important toanalyze pathogen-responsive gene expression in resistantand susceptible wheat lines, as it may increase ourunderstanding of the molecular mechanism of *Bipolaris*infection and the role of gene expression in spotblotch resistance.Interaction study experiment can help us to understand the plant response against fungal pathogen. With this intension, susceptible (DDK 1025) and moderate resistant (Chirya 3) plants were grown under spot blotch specific conditions in green house. Protein extraction method was optimized to get the good protein yield from perm gram of collected tissue sample and isoelectric focusing method conditions were also optimized. TCA-acetone extraction method was reported as best method for protein extraction from Spot blotch samples. The differentially expressed protein spots from the 2D –PAGE can be subjected to mass spectrometry analyses and candidate resistance genes can be identified for their role in interaction and/or resistance mechanism. Thefunctional genomic approach including transcriptomics and RT-PCR need to be explored andemployed on larger scale to mitigate the problems ofspot blotch disease in wheat. Integrating conventionalbreeding, molecular approach, need based applicationof fungicides and cultural options will offer eco-friendlyand cost effective control of this disease in different partsof the world.

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