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Abhishek Kumar
A) School of Biological Sciences, National Institute of Science Education and Research (NISER)-Bhubaneswar, Jatni, Odisha, India,
B) Homi Bhabha National Institute, Training School Complex, Anushakti Nagar, Mumbai, India

Gohar Taj
A) Department of Molecular Biology & Genetic Engneering College of Basic Science and Humanities, Bhubaneswar, Odisha, India
B) GB, Pant Univ. of Agric. & Tech., Pantnagar, Distt. Udham Singh Nagar, Uttarakhand, India

Anil Kumar
A) Department of Molecular Biology & Genetic Engneering College of Basic Science and Humanities, Bhubaneswar, Odisha, India
B) GB, Pant Univ. of Agric. & Tech., Pantnagar, Distt. Udham Singh Nagar, Uttarakhand, India

Correspondence
Abhishek Kumar
A) School of Biological Sciences, National Institute of Science Education and Research (NISER)-Bhubaneswar, Jatni, Odisha, India,
B) Homi Bhabha National Institute, Training School Complex, Anushakti Nagar, Mumbai, India

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Host factors of wheat altered the temporal protein expression of *Tilletia indica* in wheat

Abhishek Kumar, Gohar Taj and Anil Kumar

Abstract
*Tilltia. Indica***,** the causal agent of Karnal bunt in wheat exhibits very high variation amongst its monosporidial cultures. This variability depends upon many more factors. The wheat spikes host factors is comprised with multiple components which have profound effects on the pathogenesis in wheat. It has been also shown that genes are also involved in many more signaling pathways in the various fungi. Disruption of the gene, PMK1 of *Magnaporthe. Grisea* reduced appressoria formation and lost the ability to infect through a wound. Similarly, the CMK1 gene from *Colletotrichum lagenarium* could compliment a PMK mutant of *M. grisea* and could restore its pathogenecity. The genes codes for protein in pathogenesis it has been also shown by modules of MAP kinase signal transduction pathway. However, it is not clear how the host factors influences the temporal protein expression pattern in *Tilletia indica.*
Here, we have shown that the host factors influences the temporal protein expression pattern, in three different monosporidial strains (KB3, JKmsb and KBPN6) of *Tilletia indica.* This new finding will open up more dip understanding of pathogenesis in *Tilletia indica* in wheat. Therefore this could be useful for design the molecular marker specific to altered protein to identified specific strain of *Tilletia indica*.

Keywords: Host factors, *Tillia indica*, molecular temporal expression and pathogenesity.

Introduction
In India, the wheat is affected by a number of diseases out of which rusts, smuts, powdery mildew, loose smut, leaf blight and Karnal bunt continue to be the limiting factor in increasing wheat yield. Karnal bunt *(Tilletia indica)* is a fungal disease that affects wheat, durum wheat and triticale. The disease progresses systematically to other florets within the spikelet initially infected and then to adjacent spikelets, including those on the alternate side on the rachis (Dhaliwal, *et al*., 1983, Bedi and Dhiman 1984, Warham and Cashion 1984) [21].
It has been reported that *T. indica* against wheat shows tissue specificity towards the florets just before the anthesis (Kumar *et al.*, 2000) [9]. According to Bains (1994) [1] sporidia landing on spikes, immediately after their emergence from boot leaf, cause more infection than sporidia landing at the other stage of spikes.
The infected grain emits a rotting fish odour due to trim ethylamine secreted from teliospores and the wheat products from severely infected grain are unpalatable (Sekhon *et al.*, 1980; Singh and Bedi, 1985) [19]. Once the spikelet gets infected by the pathogen, the mycelium moves within the ear head infecting the adjoining spikelets and produces partially infected grains (Dhaliwal *et al.*, 1983) [7-8].
Because of the relatively aggressive nature of the disease and its reliance on rather exact weather conditions for infection, it is surmised that it is possible this disease has long period of latent survival between initial arrivals and becoming thriving, established disease (Marshal *et al.*, 2003) [10].
Karnal bunt disease reduces both quality and quantity of wheat grains. A direct relationship between intensity of Karnal bunt infection and reduction of wheat seed germination has been established by several workers (Singh, 1980; Singh *et al.*, 1981, Bedi and Meeta, 1981; Bedi *et al.*, 1981; Krishna and Singh, 1982). The infected grains, which retained their viability, produced higher proportion of abnormal seedlings compared with the healthy grains. However, Warham (1990) reported that infection had very little effect on seed viability, irrespective of age of the seed and that infected seeds have a lower survival rate in storage compared with

Healthy seeds of the same seed lot.

It was reported that wheat lots with 1 percent or more infected grains reduce the palatability of food products due to fetid smell and perceptible discoloration (Mehdi *et al.* 1973) [12]. Others, however, tend to agree that with 3 percent or less infected grain effect the characteristics, such as appearance and palatability of bread cookies are unaffected (Sekhon *et al.*, 1980a, Sekhon *et al.*, 1980b; Medina, 1985) [11].

Yet, the reports by Rai *et al.*, (1991) that feeding of Karnal bunt infected grains to albino rats was not safe as in such rats liver and renal insufficiency was observed which calls for long term expert studies. It was found that no known mycotoxins or ergot alkaloid present in infected wheat (Bhat *et al.*, 1980) [5]. However, it was reported that invasion of secondary pathogens on wheat grains, damaged by Karnal bunt, including *Aspegillus flavus*, which produce aflatoxins (Bedi *et al.*, 1981) [3-4]. Karnal bunt is an important issue in wheat trade, as importing countries insists on zero tolerance levels for fear of introducing the disease. According to him, Karnal bunt covered about one third area under wheat cultivation in India and was responsible for an annual loss around, 40,000 metric tons of grain per year. Rai and Singh (1985) [13-19] found that wheat cultivar UP-262 grains from ear with low, medium and high grades (extent of grain converted into bunt sorus) of Karnal bunt infection were lighter in weight by 5.5, 20.0 and 51.6 percent respectively. This amounted to an average of 25.6% or roughly ¼ of reduction of weight in a lot having all three grades of infections in equal proportion. Thus loss in yield could be interpreted as actual yield x percentage infected grain. It has been estimated that 1.0 percent of the value of wheat crop was lost due to KB during epidemic year of 1987 in Uttar Pradesh, on account of grain quality and yield losses.

Mitogen activated signal transduction pathways play a crucial role (Sophie, *et al.*, 1996) in development of virulence levels in pathogens. Through mitogen activated protein kinase (MAPK) pathways pathogens respond to external stimuli and alter their own features such as cell wall integrity, mating, morphological transition, adaptation to stress factors and this modification can leads to generate different virulence levels in phyto pathogens.

2. Materials and methods

2.1 Collection of plant materials

The wheat seeds variety WH-542 a Karnal bunt susceptible genotype was collected from Punjab Agricultural University, Ludhiana (Punjab).

2.2 Collection of plant pathogenic fungal strains

Karnal bunt KBPN6 strain was collected from G.B. Pant Uni. of Agriculture and Technology, Pantnagar. KB3 and JK (JKmsb) base isolate were collected from Delhi zone and IARI New Delhi.

2.3 Preparation of Acetone Extracts from wheat spike

Acetone extract was prepared from susceptible (WH-542) wheat (spike) variety in boot emergence stage (S_2). 50 g of plant were ground in liquid nitrogen to a fine powder using pestle and mortar. fine powder were suspended in cold acetone (1g in 10 ml of acetone) and suspension was agitated in cold condition for 5 hours and filtered through muslin cloth and stored at 4^0C in tightly capped bottles. The acetone was evaporated at room temperature using flash evaporator. Dried material was re-suspended in 1/10th of the volume of sterilized distilled water and filtered through 0.22µ filter.

2.4 Media Culture and mycelium harvesting of *Tilletia indica*

The *Tilletia indica was* cultured on both solid and liquid modified potato dextrose media PDB Broth: 24 g, Glycerine: 10 g, KH2PO4 0.1 g, Mg SO4: 0.5 g, NaCl: 50mg, Yeast Extract : 5.0 g. The volume was adjusted up to one liter and aliquoted in to 50 ml in 250 conical flasks and autoclaved. 50 ml of liquid media was transferred to 250 ml conical flasks and autoclaved. For solid PDA media, preparation was same except incorporation of solidifying agent (2% agar). The mycelial discs were inoculated in10.0 ml autoclaved PDB. The cultures were incubated in BOD incubator at 22$\pm$ 2^0C under light and dark conditions. *T. indica* were harvested at 7, 14 and 21 days' time intervals. The mycelial mat was filtered through a muslin cloth and washed in PBS followed with sterilized distilled water. The wet weight of the mycelial mass was taken and the wet masses were lyophilized for 5 hours to obtain the dry weight. Dry mycelial masses were stored in -20^0C. For the solid culture Petri plates were inoculated with fungal discs and incubated under humidified light and dark conditions at 22$\pm$ 2^0C in BOD.

2.5 Treatments of culture with Acetone extracts

The fungal isolates (KB3, KBPN6, and JKmsb) were cultured in liquid media with Karnal bunt susceptible wheat (WH 542) extracts at 1: 10 extract media ratio.

2.6 Preparation of fungal extracts

The harvested mycelial mass were kept at -20^0C for overnight. The freezed mycelia mass was ground in mortar and pestle in extraction buffer under cold conditions. The protein extracts was centrifuged at 15,000 rpm for 20 minutes. The supernatant was slowly decanted in an Eppendorf tube and stored at -20^0C for protein estimation.

2.7 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Buffers and stock solution:

(a) 2x samle buffer (pH 6.8): Tris Base 1.51 g, SDS 8.0 g, Glycerol 30.0 ml, Bromophenol blue 2.0 mg, EDTA 0.10 g**,** BME 5.0 g. All above mention components dissolved in 64 ml of distilled water. Concentrated 1M HCL added drop wise and pH was adjusted to 6.8. The volume was made up to 100 ml and stored at 4˚c.(b) Acylamide & Bis-acylamide Stock Solution: Acylamide:29.2 g, Bis acylamide 0.8 g, dissolved in 100 ml of distilled water and stirred by magnetic stirrer. It was filtered and stored at 4˚c in a dark colored bottle.(c) 2X separating gel buffer (pH 8.8): Tris base 45.40 g, dissolved in 460 ml of distilled water, concentrated HCL was added drop wise until pH fell to 8.8.Volume was made up to 500 ml and stored at 4˚c.(d) 2X Stacking gel buffer (pH 6.8): Tris base 6.0 g**,** dissolved in 70 ml distilled water and 1 M HCL were added drop wise until pH fell to 6.8.Stored at 4˚c.(e) 10% ammonium per sulfate (APS): 0.1 g APS, dissolved in1ml distilled water and used freshly prepared.(f) 10% SDS: 10.0 g SDS dissolved in 100 ml of distilled water. (g) 1X electrophoresis buffer (pH 8.3): 3.30 g Tris base, 14.4 g Glycine, 1.0 g SDS dissolved in 885 ml of distilled water. The pH was adjusted to 8.3 with 1M HCL and made volume 1 litre. (h) Staning solution: Solution I composition: 0.25 g Commassie brilliant blue R, dissolved in 25 ml distilled water. Solution II composition: 60 g Trichloroacetic acid, 180

ml Methanol, 60ml Glacial acetic acid. Solution 1 was added to solution II and mixed by stirring. Make up volume 1 litre with distilled water. (ii) Destaining solution: 70 ml Acetic acid**,** 45 ml Methanol, mixed and volume was made up 1 litre with distilled water.

2.8 Gel and sample preparation
The vertical slab gel unit was assembled in casting mode with 1.5 mm spacers. The resolving gel solution was prepared according to the given composition.30 µl of the sample was mixed with equal volume of 2X sample buffer and boiled for 5 minutes in a water bath. The wells were filled with electrode buffer. The samples and were underplayed in each well except one well side markers protein. The electrophoresis unit was connected to the power pack.

2.9 Staining and Distaining
The run gel was stained with Coomassie Brilliant Blue for two hours. Later, the gel was distained in distaining solution with intermittent shaking.

(a) Composition of Protein extraction buffer1
Extraction buffer (100 ml; pH 7.5): Tris base 0.06g, EDTA (Na2) 1.861g, EGTA 0.38g,TritonX-100 0.5g, BME 0.3g, PMSF 34.8g, Ascorbic acid 0.39g, Polyvinylpyrolidone 0.4g. P^H adjusted to 7.5 with 1N HCl and volume was adjusted 100 ml with distilled water**.** Storage at 4^0C. The harvested mycelial mass were kept in -20^0C freezer overnight. The freezed mycelia mass was ground in mortar and pestle in protein extraction buffer (1 mg wet mycelium/1 ml extraction buffer). The protein extracts was taken in centrifuge tube and centrifuged at 15, 000 rpm for 20 minutes. The supernatant was slowly decanted in an Eppendorf tube and stored at -20^0C for protein estimation, free carbohydrate estimation.

(b) Stock Solution of PMSF
(Phenylmethylsulfonyl fluoride) 174mg/in 90% ethyal alcohol.

3.10 Purificaton of Mycelial protein with Acetone precipitation
The required volume of acetone was kept at -20c. The protein sample was placed in acetone compatable tube. The four times the sample volume of cold acetone to the tube. The tube was vortexed and incubated for 60 min at -20c. The tube was centrifuged for 10 min at 13000xg. The supernatant was decanted properly. The acetone was allowed to evaporate from the uncapped tube at room temperature for 30 minute. The protein extraction buffer was added and dissolved the protein by vortexing it.

3.11 Estimation of protein
Protein detection was carried out of fungal extracts from mycelium at time intervals (7, 14 and 21 days after inoculation). The total protein content was determined by Bradford method (1976) [6]. Comassiie Brilliant Blue –G250 (CBBG-250) was used as dye for protein estimation.

(a) Dye preparation
Coomassie Brilliant Blue-G-250 (10 mg) was dissolved in 5ml of 95% ethanol; 10ml of 85% (W/V) orthophosphoric acid and diluted to 100ml. Final composition of the reagent was 0.001% (CBBG-250) 4.7% ethanol and 8.5% phosphoric acid. The formation of blue froth while shaking the Bradford reagent confirms that the solution is ready for use.

(b) Stock solution of BSA
By mixing of 0.1 mg BSA in 1ml distilled water. Different concentrations of BSA were taken in test tubes and volume was made up to 0.5 ml by distilled water. Blank was prepared by using 0.5ml distilled water and 5 ml of dye. After making the volume up to 0.5ml, 3ml of Bradford reagent was mixed to each test tube. The absorbance of each was read at 595nm after 30 min, in visible range in ELICO-SL150 UV-VIS spectrophotometer. Eight concentration of BSA viz., 5µg, 10g, 20 g, 30 g, 40 g and blank were used whose absorbance were recorded. Standard curve was plotted and protein estimation for fungal extracts was done using the standard curve.
3ml of Bradford reagent, 40ul of plant extracts/fungal extracts/dialyzed spent medium and 260ul of double distilled water were added in one reaction tube and absorbance was taken at 595nm.The protein sample of *T. indica* were elecrophoresed under reducing and denturing condition.

3.12 Determination of molecular weight of ploypeptides
After Destaining of gel the molecular weight of polypeptides were determined by the molecular weight analysis tool of the gel documentation system. The molecular weight of protein was determined by standard protein molecular weight markers (Fermantas).

3. Result and Discussion
3.1 Temporal expression of protein profiling after the 7 days of host factors
In order to assess the alteration of proteins expression of three monosporidial culture (KB3, JKmsb and KBPN6) of *T. indica* in liquid culture, the soluble protein were extracted from wet mycelia mat grown at after 7 days in presence and absence of host factors and were subjected to electrophoresis separation by SDS-PAGE under reducing condition. The 5 bands (97.4 KDa, 50 KDa, 40 KDa, and below 29 KDa) were clearly visible in presence of host factors in KB3 monosporidial culture when compared with absence of host factors where only 3 faint bands were identified. But in JKmsb monosporidial culture 6 bands (97.4 KDA, 80 KDa, 68.4 KDa, and below 29 KDa) were clearly identified and 3 faint bands were readily identified, in presence of host factors, however only 4 faint bands were observed in absence of host factors. The similarly 5 clear bands (50KDa, 40KDA, 29KDa, and below 29KDa) were observed in KBPN6 monosporidial culture in presence of host factors and 5 faint bands were observed in absence of host factors.

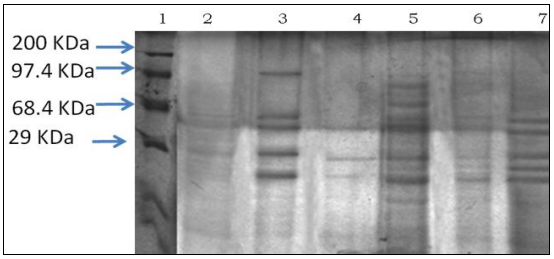

Fig 1: Identification of differential expressed protein in different strain of tilletia indica treated with host factors after 7 days. By SDS-PAGE numbering symbol 1. Molecular marker 2. KB3 NT, 3: KB3T, 4: JKmsb NT. 5: JKmb T, 6: KBPN6, NT: In absence of host factors, T: In presence of host factors.

3.2 Temporal expression of protein profiling after the 14 days of host factors

We also observed various banding pattern of protein (Fig. 3.2) of monosporidial cultures of KB3, JKmsb and KBPN6) after 14 days of growth in liqiud culture. The number of 5 bands (80 KDa, 68.4 KDa, 50 KDa, 45 KDa and 29 KDa) were intense and some faint bands observed in presence of host factors in KB3 monosporidial culture as compared to absence of host factors where only 3 faint bands (80 KDa, 68.4 KDa and 50 KDa) identified as compare to 7 days gel but some faint bands also observed in both in presence of factors as well as in absence of host factors. However, in JKmsb monosporidial culture 5 bands (97.4 KDa, 80 KDa, 68.4 KDa, 50 KDa and 45 KDa) were intense in presence of host factors, however 4 clear bands and some faint bands were observed in absence of host factors. The similarly 5 (60 KDa and 30 KDa) 97.4 KDa, 80 KDa and 68.4KDa, bands and some faint bands were observed in KBPN6 monosporidial cultures in presence of host factors and 5 faint bands were also observed in absence of host factor.

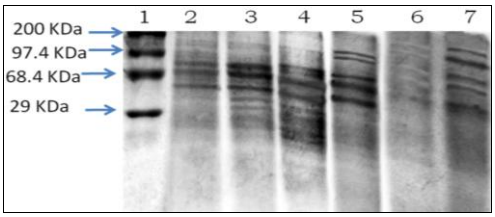

Fig 2: Identification of differential expressed protein in different strain of tilletia indica treated with host factors after 14 days. By SDS-PAGE numbering symbol 1. Molecular marker 2. KB3 NT, 3: KB3T, 4: JKmsb NT. 5: JKmb T, 6: KBPN6, NT: In absence of host factors, T: In presence of host factors.

Temporal expression of protein profiling after the 21 days of host factors

In order to understand the protein profile of 21days, grown monosporidial cultures (KB3 JKmsb and KBPN6) of *T. indica* grown in liquid culture were. The marked variation of banding pattern of protein was observed (Fig. 3.3).

The number of 5 bands (97.4 KDa, 80 KDa, 68.4 KDa, 45 KDa and 29 KDa) was clearly observed and 6 faint bands also observed inpresenceof host factors in KB3 monosporidial culture as compare to in absence of host factors where only 2 clear bands (97.4 KDa and 45 KDa) as well as 6 faint bands also identified. But in JKmsb monosporidial culture 6 bands (80 KDa, 68.4 KDa, 50 KDa 45 KDa and below 29 KDa) were identified and, in presence of host factors, however only 5 bands were observed in absence of host factors. The similarly 7clear bands (100 KDa, 97.4 KDa, 80 KDa, 70 KDa, 50 KDa, 40 KDa below 29 KDa) and were observed in KBPN6 monosporidial culture on the gel in presence of growth factors and 6 faint band were observed in absence of host factors.

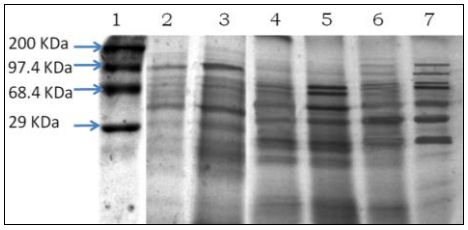

Fig 2: Identification of differential expressed protein in different strain of tilletia indica treated with host factors after 21 days. By SDS-PAGE numbering symbol 1. Molecular marker 2. KB3 NT, 3: KB3T, 4: JKmsb NT. 5: JKmb T, 6: KBPN6, NT: In absence of host factors, T: In presence of host factors.

It was found that as the monosporidial culture progressd the population of the mycelium were subsequently increased up to 14 days in some (KBPN6) monosporidial culture. However in some monosporidial culture (KB3 and JKmsb) the population of the mycelium increased up to 21days. The summary of total number of observed bands both in absence of host factors and presence of host factors mention in tabular form (table 3.1).this result clearly suggested the significantally higher number of bands observed in presence of host factors as compare to absence of host factors.

Table 1: Time kinetics changes in protein banding pattern of fungal culturs grown in presence and absence of host factors**.**

Fungal cultures	Total protein bands at different time intervals					
	7days		14days		21days	
	NT	T	NT	T	NT	T
KB 3	3	5	4	6	4	6
JKmsb	2	6	4	5	5	6
KBPN6	3	5	5	5	6	6

5. Summary and Conclusion

In summary we conclude that the protein banding pattern obtained on the SDS-PAGE for mycelial extracts grown in presence and absence of host factors. The variation associated with the differential expression of protein induced by host factors during the growth and development of the fungus. The number of bands in presence of host factors at 7, 14 and 21 days were incresed and intensified when campared with absence of host factors.

It was observed that some bands common in all time interval. The number of protein bands common at all-time intervals presences of growth factors was higher than in absence of host factors. In all the stages few unique bands were also present. Some similar bands of proteins were also observed at different time intervals. The most plausible explation for more number of bands observed in presence of host factors is due to activation of signaling pathway by host factors which causes activation of many genes and protein during morphogenetic development of fungi *T. indica.* The some common protein was observed bands during all the phase of development. This shows that few proteins remained present during all the phase of development. Fungi employ signaling pathway in many process including the control of differentiation, sexual development and virulence in addition to the monitoring of nutrional status and stress.

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