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Host factors induces immunomodulator effect on pathogenicity of *Tilletia indica* in wheat

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Abstract
Wheat (*Triticum aestivum* L.) is one of the most important staple foods of the India or even in world. The food grain production of wheat is affected by many more diseases however; the Karnal Bunt (KB) disease is an important disease and caused by *Tilletia indica.* In this study we have taken three important monosporidial strains of *Tilletia indica* such as KB3, JKmsb and KBPN6 and they can easily differentiate by each other by morphological variation. Host factor(s) extracted from wheat spikes are heat labile, comprised with multiple components which produce nutritional as well as hormonal effects on the pathogen. It is quite evident that hrost factor(s) trigger the MAP kinases machinery in order to provide signal for induced mycelination, formation and alteration of genetic and surface properties. However it is not clear how the pahtogenisity induces by *Tilletia indica* in wheat.
Here we have shown that host factor(s) treatment induces the pathogenesis of *Tilletia indica* in wheat by altering the expression pattern of immunodominant proteins. We also observed the time kinetics variation of protein expression in the presence of host factors. Our this study alteration of protein expression pattern in the presence of host factors opens up a possibility of understanding the onset of disease in more detail and that might be helpful to manage the Karnal bunt disease of wheat.

Keywords: Pathogeneity, *Tilletia indica*. Host factors, immunodominant proteins

1. Introduction
Wheat (*Triticum aestivum* L.) is one of the most important staple foods for nearly 40 per cent of the world. The total area and production under wheat in India in the year 2018 was 305.97 m ha and 92.27 million tons respectively (Aspects of India's Economy, 2019) [3]. The introduction of semi dwarf high yielding varieties revolutionized the production of the crop. It is revealed that India has increased the productivity of wheat about sevens folds from around 12.26 million tons in 1964- 65 to 92.5 million tons in 2017-2018 (Economic Survey of Indian Agriculture, 2019) and the average productivity has reached up to 3216 kg/ha.
Prevalence of different diseases in wheat growing areas has been increased in coming years. Among these diseases Karnal bunt (KB) a floret influencing disease caused by the basidomycetes fungus, *Tilletia indica* (Syn. *Neovossia indica)* is very important disease especially in the north-western regions of India. It was discovered near the city of Karnal in Punjab, India (Mitra, 1931). It has been also reported to occur in Pakistan, Iraq, and Afghanistan and Nepal (Joshi *et al.*, 1983; Warham, 1986; Gill *et al.*, 1983; Singh 1984). The disease was detected in Mexico in 1970 (Warham, 1986), and southern parts of U.S.A. in 1996 (Ykema *et al*., 1996). 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). Host and pathogen interaction is very much pronounced in development of different virulent levels of *T. indica* against wheat (Kumar *et al.*, 2000) [23]. The infected grain emits a rotting fish odour due to trimethylamine secreted from teliospores and the wheat products from severely infected grain are unpalatable (Sekhon *et al.*, 1980; Singh and Bedi, 1985).
Three developmental stages were compared for susceptibility to KB and occurrence of *T. indica* sporidia on spikes at boot leaf stage (S2) were more susceptible than emerged spikes at anthesis (S3) (Bains, 1994) [5]. 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, Rai and Singh 1978) [41, 40, 42].

Results of studies on toxicological aspects of bunted grains indicated absence of acute toxicity to laboratory animals (Bhat *et al.*, 1980; Bhat *et al.*, 1983; Rai, 1983) [7]. However, (Bedi *et al.*, 1981) reported invasion of secondary pathogens on wheat grains, damaged by Karnal bunt, including *Aspegillus flavus*, which produce aflatoxins.

Naturally, the disease appears sporadically in isolated pockets as minor disease causing insignificant loss in yield, but in certain years it assumes epiphytic proportions and causes substantial losses in yield (Munjal, 1975; Joshi *et al.*, 1970; Aggarwal *et al*., 1976) [31]. The benefits of breeding programme and agronomic practices are limited due to meager knowledge of biology of pathogen, sexual development and its Phyto pathogenesis (Gill *et al.*, 1981). A significant reduction in yield and quality is seldom associated with this disease. However, due to its mode of dissemination and control difficulties many countries have imposed strict quarantines on the importation of infected seed. In many cases, any seed produced from an area where the disease has been identified cannot be imported.

2. Materials and Methods

2.1 Plant materials and fungal strains

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

2.2 Development of monosporidial strains of *Tilletia indica*

Sporidia were collected from base isolates and germinated on the same media. Single germinating sporidia were collected microscopically.

2.3 Preparation of Acetone Extracts

Acetone extract was prepared from spike tissues collected from susceptible (WH-542) wheat variety in boot emergence stage (S_2). 50 g of plant parts were ground in liquid nitrogen to a fine powder using pestle and mortar. Ground plant tissues 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 ºC in tightly capped bottles. The acetone was evaporated at room temperature using flash evaporator. Dried material was re-suspended in $1/10^{th}$ of the volume of sterilized distilled water and filtered through 0.22µ filter.

2.4 Media Preparation

The *Tilletia indica was* cultured on both solid and liquid modified potato dextrose media. 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).

2.5 Culture of *Tilletia indica*

The mycelial discs were inoculated in10.0 ml autoclaved potato dextrose broth. The cultures were incubated in BOD incubator at 22+ 2 ºC under light and dark conditions. The growing liquid cultures of *T. indica* were harvested at desired time intervals. The media containing the mycelial mat was filtered through a muslin cloth and washed several times 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 ºC. For the solid culture Petri plates were inoculated with fungal discs and incubated under humidified light and dark conditions at 22+ 2 ºC in BOD.

2.6 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.

2.7 Preparation of fungal extracts

The harvested mycelial mass were kept at -20 ºC 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 ºC for protein estimation.

2.8 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Buffers and stock solution

a) **2x sample 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 at magnetic stirrer so as to dissolve it completely. It was filtered and stored at 4 ˚C in a dark coloured 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 dropewise 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 dropwise until pH fell to 6.8.Stored at 4 ˚C.
e) **10% ammonium persulfate (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.
i) **Destaining solution:** 70 ml Acetic acid**,** 45 ml Methanol, mixed and volume was made up 1 litre with distilled water.

2.9 Gel 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.

2.10 Sample Preparation
30 µl of the sample was mixed with equal volume of 2X sample buffer and boiled for 5 minutes in a water bath.

2.11 Loading and running the gel
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.

2.12 Staining and Distaining
The gel was disassembled after completion of the run and gel was stained with Coomassie Brilliant Blue for two hours. Later, the gel was distained in distaining solution with intermitant shaking.

2.13 Immunological Identification of Immobilized Proteins: Western Blotting: "Western" bloting has been done by according to Towbin *et al*. 1979) and Gershoni & Palade 1982, Renart & Sandoval 1984) [18].

2.14 Buffer Stock Solutions
a) **Transfer buffer (pH 8.3):** Dissoleved Tris base (5.8 g), Glycine (2.9 g), SDS (0.37g) and Methanol (200 ml). in distilled water and volume was make up 1litre
b) **Tris NaCL Buffer (pH 8.0):** 10X. **(**Dissolved in distilled water and ajusted the pH 8.0 with 1.0M HCL. to makeup 500 ml.
c) **Serum dilution Buffer:** 100 ml: Bloking buffer (20.0 ml), Wash Buffer 80.0 ml.
d) **Phosphate -buffer saline (pH 7.4):** Dissolved the NaCl (8.0 g), KCL (0.2 g), Na2HPO4 (1.44 g) and KH2PO4 (0.24 g). in 800 ml of distilled water. Adjusted the pH 7.4 with HCL and volume was made up to 1litre.
e) **Alkaline phosphatase:** The substrate 5-bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium (BCIP/NBT) is converted in-situ into a dense blue compound by immunolocalized alkaline phosphatase.

When the SDS- polyacrylamide gel approached the end of its run, marked one corner of the filter with a soft-lead pencil. The cuteed six pieces of Whatman 3MM paper and one piece of nitrocellulose filter to the exact size of the SDS-polyacrylamide gel. Floated the nitrocellulose filters on the tray containing transfer buffer for 5 minutes. Similarly also soaked the six pieces of 3MM paper in tray containing a transfer buffer. Layed the bottom electrode (anode) flate on the bench. Placed the electrode on three sheets of 3MM paper that has been soaked in transfer buffer. Stacked the sheets one on top of the other. Squeezed out air bubbles using a glass pipette. Placed the nitrocellulose filter on the stacked of 3MM paper. Remove the glass plates and transfer the gel into transfer buffer for 5 minutes placed the gel on the top of nitrocellulose filter and squeezed out trapped air bubble. Placed the final three sheets of 3MM paper on the gel, again exactly aliened and that no air bubbles trapped. Placed the upper electrode (cathode) on top of the stack. Connected the leads with current of 0.65mA/sq cm. of gel for a 4 hours and disconnected the leads. Transfered the gel to a tray containing Coomassive Brilliant Blue, and stained it to check whether electrophoretic transfer is completed. Removed the nitrocellulose filter from the sandwich and transferred it to a clean piece of 3MM paper.

2.15 Animals for antibody production
Polyclonal antibodies which include anti-mycelial, has been produced at the Molecular Markers Laboratory Dept. of Molecular Biology and Genetic Engineering, C.B.S.H, G.B.P.U.A. and T, Pantnagar. New Zealand white female rabbit (NZWFR) were used for production of anti mycelial antibody. These antibodies were developed in Molecular marker lab.

2.16 Chemicals
Antigoat immunoglobulin (IgG) conjugated with alkaline phosphatase; substrate BCIP/NBT and dNTP were purchased from M/s Bangalore Genei, Bangalore. All other chemicals used were of analytical grade originated from M/s Sigma Chemical Co. USA.

2.17 Blocking binding sites for immunoglobulins on the nitrocellulose filter
Placed the nitrocellulose filter in a tray and added 0.1 ml of blocking buffer and incubated the filter for 2 hours at room temperature with gentle agitation. Washed the filter 3 times with wash buffer.

2.18 Incubating the nitrocellulose filter with the antibodes
Washed filter inubated with diluted primary antibody (1:500, anti-rabbit, anti- mycelia protein) and incubated the filter for 2 hours with gentle agitation. Washed the filter 3 times with wash buffer and immediately incubated the filer with the secondary antibody, raised in goat. Transferred the nitrocellulose filter in tray containing alkaline phosphatese coupled secondary reagent 1:500. Incubated the membrane for 1 hour at room temperature with gentle agitation. Washed the incubated filter 3 times with wash buffer and added 0.1ml BCIP/NBT. Incubated the filter at room temperature with gentle agtation. Monitored the progress of the reaction carefully (about 20 minutes).Transferred the filter to a tray containing 50 ml of phosphate-buffered saline. Photographed the filter.

3. Result and Discussion
3.1 Change in protein profiling under the influence of Host Factors
In order to assess the alteration of proteins expression of three monosporidial culture such as KB3, JKmsb and KBPN6) of *T. indica* in liquid culture, the soluble protein were extracted from wet mycelia mat (30 days) in the presence of Host Factors and in absence of Host Factors. The mycelia protein extract of *T. indica* were subjected to electrophoresis separation by SDS-PAGE under reducing condition. The fungal extracts prepared were electrophoreses on the polyacrylamide gel (Fig 1). The numbers of protein bands were higher in presences of Host Factors as compared to absence of Host Factors.
The protein expression of *T. indica* in presence and in absence of Host Factors was observed. 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*. 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.

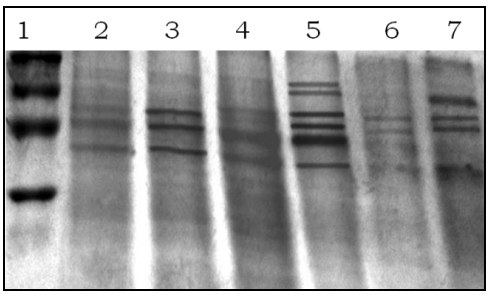

Fig 1: Identification of differential expressed protein in different strain of Tilletia indica treated with Host Factors after 30 days by SDS- PAGE.

Numbering symbol 1. Molecular marker 2. KB3 NT, 3. KB3 T, 4. JKmsb NT, 5. JKmsb T, 6. KBPN6 NT, KBPN6 NT, In absence of host factors, T In presence of Host factors

3.2 Alteration of immunodominant protein in presence of Host Factors.

In order to identify of Immunodominant proteins associated with the alteration of protein expression of three monosporidial culture (KB3, JKmsb and KBPN6) of *T. indica* in liquid culture, the soluble protein were extracted from wet mycelial mat (fig 1), in presence of Host Factors and in absence of Host Factors. The mycelial protein extract of *T. indica* were subjected by electrophoretic separation by SDS-PAGE under reducing condition. Simultaneously Western bloting has been done by using anti-mycelial antibody. It was observed that the faint bands was observed in absence of Host Factors and intense bands has been observed in presence of Host Factors in the all mono sporidial culture suh as, J Kmsb, KB3 and KBPN6) of *T. indica* (Fig 2). The crud IgG antimycelial antibodies to *T. indica* recognized unique immunoreactive bands.

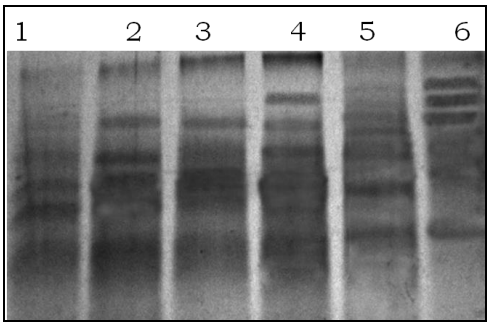

Fig 2: Differential expression pattern of immunodominant protein in different strain of *Tilletia indica* treated with Host factors after 30 days.

Numbering symbol 1. KB3 NT, 2. KB3 T, 3. JKmsb NT, 4. JKmsb T, 5. KBPN6 NT, 6. KBPN6 NT, in absence of host factors, T In presence of Host factors

The monosporidial KBPN6 mycelial protein showed more immunoreative bands as compared to KB3 and JKmsb monosporidial mycelial proteins. The three monospridial culture showed altered proteins with differential expression of immuno dominant proteins. The immunodominant protein patters also idicated the variability among the three strains in presence of host factors when compard in absence of host factors mycelial proteins. KB3 isolate also showed considerable variation similar together monosporidial cultures of *T. indica* such as JKmsb and KBPN6.

5. Conclusion

In summary, we conclude that variation associated with the differential expression of protein could be because of Host Factors. The number of bands in the presence of host factors were increased and intensified as compared with absence of host factors. The most plausible explanation is due to activating of signaling pathway by Host Factors which causes activation of many genes and proteins during the growth, and pathogenesis of *T. indica*. Host Factors treatment tends to increase the immuno-reactivity of *T. indica* strains.

The protein (gene) expression has been noticed in inducible manner by Host Factors. As the protein (gene) expression is not detected in absence of host factors. The gene expression takes place purely in inducible manner by the Host Factors. These findings reveal that the Host Factors behave as inducer the gene expression. With present findings it is possible to draw a conclusion that the proteins play a very crucial in fungal pathogenesis in *T. indica* and it is inducible though Host Factors treatment. The three strains (KB3, JKmsb, and KBPN6) of *Tilletia indica* showed enhanced growth and development because of high level of immunodominant protein expression which has been expressed in presence of Host Factors. This finding can be used as a bioassy to discriminate a unknown population of *T. indica* into their respective pathogenic levels with respect to a known Host Factor derived from susceptible variety witoght field testing.

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