Enhanced proteolysis leads to pre-mature cell death under the influence of elicitor like mycelial components from Karnal bunt (Tilletia indica) pathogen in wheat callus cultures

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Calli raised from mature embryos of susceptible wheat cultivar WH 542 were used in the present study as in vitro bioassay system to study the influence of disease determinant(s) of Karnal bunt (Tilletia indica), a semi-biotrophic fungal pathogen of wheat. Influence of elicitor and conditioned medium (CM) prepared from fungal cultures of T. indica was investigated on induction of programmed cell death (PCD). Induction of PCD was observed as hypersensitive response (HR) in terms of browning at localized regions of callus cultures and induction of proteolytic enzyme(s). Elicitor treated calli showed higher induction of protease activity than untreated and CM-treated cultures, which showed not much change in the activity. It was further substantiated by gel protease assay and activation of caspase-3 like protease(s) in callus cultures that clearly suggested the presence of signaling molecule(s) in the fungal elicitor preparation rather than in conditioned medium. This study further demonstrated that only elicitor preparation possesses such molecule(s), which might be cell wall bound components, rather than secretory in nature as CM was unable to induce PCD in wheat callus cultivars.

Keywords: Elicitor, Karnal bunt, Programmed cell death, Proteolysis, Wheat callus

Wheat production is affected heavily due to Karnal bunt (KB) in India. Being a major production constraint, KB is attracting the attention of breeders and government. At present, no cultivar of aestivum group of wheat has been found immune to KB although, they differ in the degree of susceptibility. Hence, it is realized to understand the various key components of pathogen attack and host defense.

KB caused by Tilletia indica is a semibiotrophic and floret influencing disease. Due to its semibiotrophic nature, it was thought to explore wheat callus culture system as a model for the study of enhanced proteolysis which leads to premature cell death mediated by fungal elicitor. In order to ascertain whether or not, KB pathogen possessed such elicitor (either cell wall bound or secretory in nature), an initial attempt was made to prepare elicitor (E) and conditioned medium (CM) from T. indica culture. The calli established in the present investigation, can be ideal bioassay systems, to study the effect of these fungal preparations on induction of hypersensitive response (HR) as a part of plant defense.

Hypersensitivity response related cell death is one of the defense responses; thus cell death in plant responding to pathogen occurs not only in susceptible reaction, but also in resistance responses. HR, linked with cell death require active plant metabolism and depends on the activity of host transcriptional machinery. HR cell death requires the expression of so called pathogenesis related proteins generation of reactive oxygen species, rapid influx of Ca++, production of phytoalexins and cross linking of cell wall.

Therefore, there is a need to study the elicitor mediated downstream signalling for induction of plant defenses. In the present study, an attempt has been made to identify the nature (secretory or cell wall bound) of the signalling molecule (elicitor) from KB pathogen and its origin.

Materials and Methods

Preparation of mycelial elicitor (E) and conditioned medium (CM)

Fungus Tilletia indica was obtained from Department of Plant Pathology, College of Agriculture, G. B. Pant University of Agriculture & Technology, Pantnagar. Ten discs from fungal colony
(5 × 5mm) were inoculated on 20 ml of potato dextrose medium (PDB powder 2.4%, glicerine 1.0%, KH-P0₄ 0.5%, MgSO₄ 0.05% and NaCl 0.005%) and grown on a rotatory shaker for 7 days at 22±1°C. Entire culture was autoclaved, homogenized and ultrasonicated for 1 min. The ultrasonicated material was centrifuged at 15,000 rpm for 20 min and the supernatant was autoclaved at 121°C (15 psi) for 20 min and stored at 4°C as stock of fungal elictor preparation. Only 2 ml of elictor was inoculated in 30 ml Murashige and Skoog (MS) medium. However, a fungal culture grown for 7 days were filtered through muslin cloth and then supernatant was autoclaved at 121°C (15 psi) for 20 min. The spent culture supernatant was used as conditioned medium.

Establishment of callus culture
Callus cultures were established from mature embryo of susceptible wheat (WH 542). Wheat seeds were surface sterilized with 30% sodium hypochlorite for 20 min and rinsed three to four times and kept at room temperature for 20 hr for soaking. Mature embryos were excised and inoculated on MS basal medium (~30 ml) supplemented with 2 ppm 2,4-D for callus induction in Petri plates. These cultures were incubated under dark at 22°C ± 1°C for 6 weeks. After 25 days, calli were subcultured in maintenance medium (MS medium supplemented with 100 ppm proline).

Treatment of wheat calli by elictor and conditioned medium
Calli of susceptible cultivar were maintained in the medium containing elictor and conditioned medium at the concentration of 2ml of elictor/30 ml of medium (6.6% V/V). These calli were observed at 5, 15 and 30 days interval. The calli were harvested and total soluble protein was extracted as follows from these calli for determination of protease and caspase-3 like proteins.

Determination of enzyme activities in elictor and conditioned medium treated wheat calli

Extraction of total soluble protein—Proteins were extracted by grinding 1.0 g of elictor (E) and conditioned medium (CM) treated cultured callus in 1 ml of extraction buffer [20mM, Tris HCl; 20mM, NaCl; 1mM, EDTA and 0.5% PVP (pH 7.8)] with an autoclaved pestle and mortar. The slurry was centrifuged at 10,000 rpm for 20 min at 4°C with Sigma 3K18 centrifuge. The supernatant was filtered through Whatman's filter paper and stored at -20°C.

Protein concentration was determined in different samples by Bradford method using bovine serum albumin (BSA) as a standard protein.

Determination of protease activity by semi-quantitative method—The agar medium containing gelatin (0.12%) was prepared in 50 mM, Tris HCl and poured in Petri plates. The wells were cut and after sealing of the bottom each well were loaded with 30 μl of protein sample. The plates were, then, incubated overnight at 37°C. After incubation, the clear zone around each well was observed. The area of activity zone was compared with that of trypsin activity zone and their activity was determined by standard curve in terms of units.

Protease activity test by tyrosine release—For quantitative estimation of protease activity, 100 μl of different protein samples were added in microfuge and then in each tube 15 μl of 10mM CaCl₂ was added, after which 250 μl of 50mM Tris-HCl (pH 7.5) buffer followed by 250 μl of 0.12% gelatin (substrate) was added. All the tubes were kept at shaking rate 200 rpm and incubated at 37°C for 4 hr. After incubation 450 μl of TCA (20%) was added in each tube. All the tubes were centrifuged at 4°C, 10000 rpm for 10 min and supernatant was taken and the tyrosine concentration was determined by Lowry method using tyrosine standard curve. From tyrosine level, the specific activity of protease in different protein samples was calculated. The specific activity of protease was amount of protease that could release 10 μg of tyrosine per mg protein/hr under standard assay condition.

In gel protease assay—Separation of total soluble proteins by SDS - PAGE was performed on 10% separating gel with 0.12% gelatin (Chacks and Springhorn 1980) and 5% stacking gels (30 : 0.8; acrylamide : bis-acrylamide).

A 40 μl aliquot of each sample was loaded on each well. Electrophoresis was performed at 100v for 2 hr. The gel was fixed and stained in staining solution consisting of methanol (25%), acetic acid (7%) and Coomassic Brilliant Blue G250 (0.1% w/v) for overnight and then gel was kept in destaining solution (7.5% acetic acid and 5% methanol) for 8 hr, observed the bands and estimated the molecular weight by electrophoresis using molecular weight marker proteins consisted of a mixture of proteins with different molecular weights i.e. 97,900, 66,000, 48,000, 20,100, 1,43,000 kDa.
Determination of caspase-3 like protein(s) by dot immuno assay—The protein samples prepared as described earlier were spotted on nitrocellulose membrane strip and allowed to dry. The nitrocellulose strip was blocked by immersion in 2% bovine serum albumin for 1 hr at room temperature for non-specific protein binding. The membrane was then rinsed with fresh phosphate buffer saline twin-20 (PBST) for 2 min, then 10 min and finally with 5 min agitation. The membrane was then, probed with rabbit anti-human caspase-3 procured from Santa Cruz Biotechnology Inc, USA, and subsequently, processed for development of dots using goat anti-rabbit secondary antibodies conjugated with alkaline phosphatase and a precipitable substrate BCIP/NBT (M/s Banglore Genei, India).

Results and Discussion

Induction of cell death in terms of browning at localized regions was observed in the presence of elicitor. Browning was initiated after 5 days of growth and continuous increase in browning was observed at later stages of callus growth. However, calli growing in the medium supplemented with CM, showed no sign of browning and calli growth was similar to that of control (Fig. 1 a-d). The study clearly revealed that signalling molecules required for induction of HR-like response in callus culture was present in E preparation rather than in CM preparation of fungal culture.

It was presumed that elicitor (E) preparation contained signal(s) for elicitation of HR and were cell wall bound fragments rather than secretory compounds as CM contained that did not induce HR like response. These fragments from the fungal cell wall were released at the site of contact with the host cell. This could be explained in the present study as elicitor was prepared by autoclaving of fungal cultures and crushing of fungal cell walls by homogenization that elicited HR and cell death in callus culture.

Activation of plant defense responses by a variety of fungal elicitors appears to involve a sequential activation of cell death pathways. With respect to association of apoptosis, a common phenotype of HR, the morphological markers of apoptosis have been reported in lesions associated with HR1,5,10,11. This suggests that the activation of PCD pathways may be involved in HR resistance response. Regardless of whether PCD pathways are co-opted in HR, it remains to be proven that cell death has a determinative role in resistance. In order that HR should occur as a form of programmed cell death, a cascading activation of a family of cysteine protease known as caspases should occur. Since plants can produce a variety of proteolytic enzymes, it is likely that some types of proteases are also involved in elicitor mediated cell death12. In order to establish the role of protease/caspase-3 like proteins in elicitor induced HR, the protease activity was determined in different calli grown both in the presence or absence of elicitor at 15 days of growth.

Enzyme activity was monitored using semi-quantitative and quantitative determination of resistance.

![Fig. 1](image_url)
proteolytic activity, in-gel protease assay and also immunological detection of caspase-3 like proteins. The higher induction of proteolytic activity was observed in elicitor treated calli than control calli and CM treated cultures, which showed no sign of HR (Table 1). Determination of protease activity by semi-quantitative method indicated that diameter of hydrolytic activity zone was more in the elicitor treated calli than its controlled calli (Table 1) and calli treated with CM. Pattern of protease activity by quantitative method was similar to that of semi-quantitative method (Table 2). Induction of protease was consistently associated with the localized plant cell death caused by treatment of elicitor. It was further substantiated by in-gel protease assay which clearly demonstrated the induction of various types of proteases in elicitor treated calli that were visible in the form of distinct band. The proteolytic bands were not observed in control calli and CM treated calli (Fig. 2). In response to pathogen invasion, a plant shows two types of cell death responses. If the plant is resistant to the pathogen, a rapid cell death is frequently triggered at the primary site of infection which constitutes the hyper-sensitive response and is accompanied by activation of local defense responses. If the plant is susceptible, disease develops, and slower cell death is initiated as local and systemic infection. At present, little is known about the execution processes that lead to PCD in plant and involvement of specific proteases, caspase-like protease.

Table 1—Determination of protease activity due to effect of E and CM in wheat calli raised from susceptible wheat cultivar at 15 days of growth by semi quantitative method

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hydrolytic zone of gelatin (diameter in cm)*</th>
<th>Equivalent trypsin units/10 µg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.9</td>
<td>11</td>
</tr>
<tr>
<td>Elicitor</td>
<td>1.2</td>
<td>27</td>
</tr>
<tr>
<td>Conditioned Medium</td>
<td>1.0</td>
<td>16</td>
</tr>
</tbody>
</table>

*Each value represents the mean of three independent determinations

Induction of caspase-3 like proteins in elicitor induced HR demonstrated in the present study employing heterologous antibody probe against human caspase-3. Due to conserved nature of caspase protein in both animals and plants, immunological evidences suggested their possible involvement in the elicitor mediated HR and PCD in callus cultures (Fig. 3). For determination of induction of Caspase-3, 2 µg of different protein samples were loaded on nitrocellulose membrane in duplicate and detected by DIBA using alkaline phosphatase conjugated goat anti-rabbit IgG as second step reagent. It was found that induction of caspase-3 like protein was observed in elicitor treated calli, which was indicated by blue colour dot. No blue colour dot was appeared in control and culture filtrate treated calli. Induction of caspase-3 like protein could be interpreted on the basis of induction of premature cell death through activation of caspase dependent pathway in which senescence proteins might be released from precursor cell death proteins that ultimately enhanced proteolysis and cell death. Further studies are needed.
to discriminate the role of specific proteases in different types of cell death responses needed for susceptibility and resistance mechanism.

The studies are underway to characterize the molecular nature of elicitor derived from KB fungus. Beside it, molecular studies of elicitor mediated downstream signaling for induction of plant defense and PCD is required to understand the pathogenesis and resistance mechanism of Karnal bunt.

References