

## Immunolocalization of sorghum antifungal protein in embryonic seed tissues

Plants have several inherent inducible defence mechanisms like lignification, phytoalexin production, hypersensitive reaction and production of defence-related proteins that act to limit pathogen infection<sup>1</sup>. The defence-related proteins are ubiquitously produced to protect against microbial attack<sup>2</sup>. Seeds contain several groups of antifungal proteins like chitinase, glucanase and non-enzymatic, low molecular weight proteins to protect the developing embryo from infection<sup>3,4</sup>. Such proteins have been identified from maize<sup>5</sup>, barley<sup>6</sup>, flax<sup>7</sup>, radish<sup>8</sup>, *Amaranthus caudatus*<sup>9</sup>, *Mirabilis jalapa*<sup>10</sup>, *Helianthus annuus*<sup>11</sup>, tomato<sup>3</sup>, sorghum<sup>12-14</sup> and onion<sup>15</sup>. Generally, these proteins are not race- or species-specific and have a broad spectrum of activity. Identification of such proteins would lead to isolation of genes that have tremendous potential in developing transgenic plants with disease-resistant trait.

Several classes of antifungal proteins, including chitinase, glucanase, sormatin, proteinase inhibitors, ribosome inactivating proteins and thionins were reported in seeds of sorghum<sup>14,16-19</sup>. We had earlier reported the presence of four uncharacterized antifungal proteins of molecular weight 20.5, 16.3, 13.9 and 12.2 kDa from mature seeds of *Sorghum* showing toxicity to aflatoxigenic isolates of *Aspergillus flavus*<sup>13</sup>. The 20.5 kDa protein was found to be more potent than the other proteins, since it inhibited the spore germination of *A. flavus* at a concentration of 10 µg/100 µl of assay media, while the 16.3 and 12.2 kDa proteins inhibited at a higher concentration of 15 µg/100 µl. The 13.9 kDa protein showed partial inhibition under *in vitro* conditions<sup>20</sup>. The present study was carried out to localize the 20.5 kDa protein in the seeds of *Sorghum bicolor*.

Seeds of *S. bicolor* variety B48826 were obtained from International Crops Science Research Institute for the Semi-arid Tropics (ICRISAT), Patancheru. Polyclonal antiserum was raised against the 20.5 kDa antifungal protein in New Zealand white rabbits. The purified protein was suspended in phosphate-buffered saline (PBS; 0.02 M sodium phosphate buffer, pH 7.2 with 0.14 M NaCl) and an emulsion was prepared using equal vol-

ume of Freund's complete adjuvant (Bangalore Genei Ltd, Bangalore). The emulsion was injected subcutaneously into the arms and legs of the rabbit<sup>21</sup>. Subsequently, on the 28th day, a booster dose of alum-precipitated protein was injected and the rabbit was bled on the 35th and 42nd days. After removal of the clot by centrifugation, the serum was stored in aliquots at -70°C. The antibody titre was tested by immunodiffusion with the pure antigen.

The purified protein was separated on 10% SDS-PAGE. The gels were blotted onto nitrocellulose filter in Pharmacia-LKB, Novoblot Multiphor unit for 2 h in a transfer buffer (39 mM glycine, 48 mM Tris, 0.0375% (w/v) SDS and 20% (v/v) methanol). After blotting, the membrane was floated on TBST buffer (10 mM Tris HCl, pH 8.0; 150 mM NaCl; 0.05% Triton X 100). The non-specific protein-binding sites were saturated by incubating the membrane in blocking solution containing 1% BSA (w/v) in TBST buffer for 30 min with gentle agitation. The blocking solution was replaced with TBST containing primary antibody in appropriate dilution (1 : 300). The unbound antibody was removed by washing the membrane in TBST three times (10 min each). The membrane was then transferred to TBST containing goat-anti-rabbit IgG-alkaline phosphatase (AP) conjugate (Bangalore Genei Ltd) at the dilution of 1 : 500 for 30 min with gentle agitation. The membrane was then washed thrice in TBST (10 min each) and subsequently transferred to AP colour development solution (Bangalore Genei Ltd). After the colour developed, the membrane was washed several times in deionized water and air-dried and stored at 4°C.

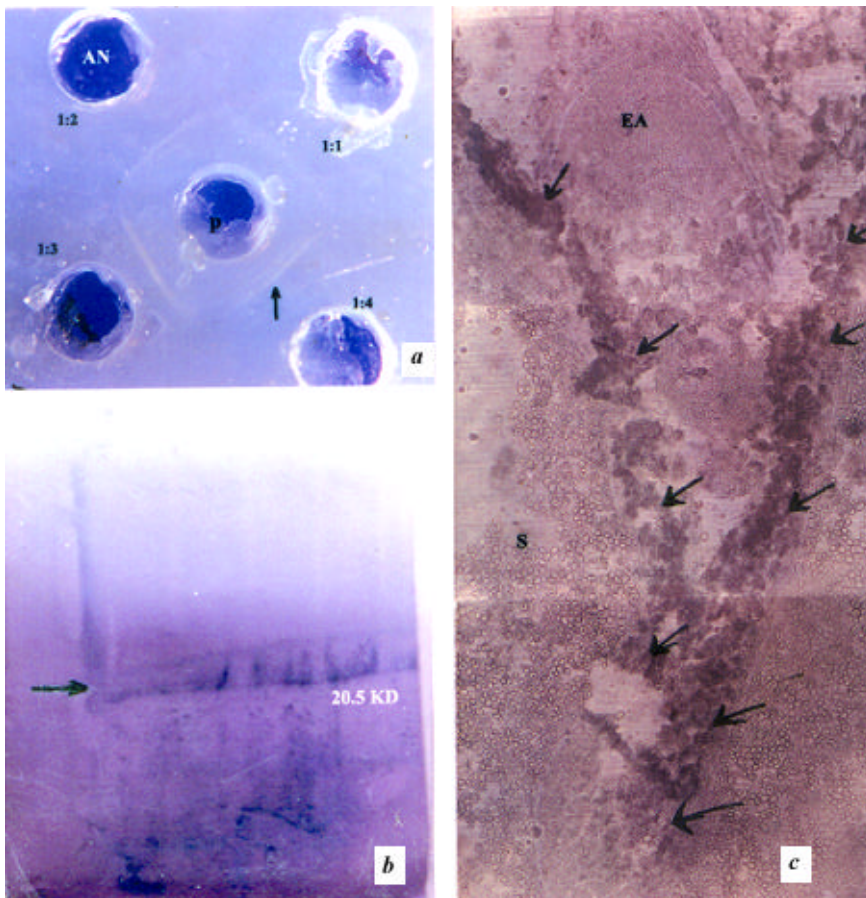
Immunolocalization study was done to determine the protein localization in sorghum seeds. The developing seeds (30 days after anthesis) of *S. bicolor* variety B 48826 were fixed in 4% (v/v) formaldehyde-PBS for 72 h at room temperature. The seeds were dehydrated in a gradual series of alcohol solutions followed by xylene incubation before infiltration and embedding in paraffin wax. Sections (10 µm thickness) were cut in a sandon rotary microtome. The sections

were deparaffinized with xylene followed by rehydration in decreasing concentrations of ethanol, distilled water and finally PBS. The sections were placed in 0.3% hydrogen peroxide in PBS for 20 min. Unspecific binding of antibodies was prevented by incubating sections in PBS containing 4% BSA for 1 h. The sections were then incubated in PBS with 4% BSA containing primary antibody (1 : 300 dilution) for 1 h, followed by three rinses in PBS. Sections were later reincubated for 30 min in PBS containing 4% BSA and goat anti-rabbit IgG-AP conjugate. The sections were again rinsed thrice in PBS followed by incubation in AP colour development solution. Counterstaining was done using safranin, followed by a 10 min rinse in distilled water. Sections were mounted in distilled water and photographs were taken in Leitz Wetzlar microscope.

We had earlier reported the purification of four low molecular weight antifungal proteins from sorghum seeds<sup>13</sup>. One of the proteins (20.5 kDa), showing maximum toxicity towards aflatoxigenic *A. flavus*, was used to raise polyclonal antiserum in New Zealand white rabbits. The antibodies reacted specifically with the antifungal protein in immunodiffusion assay (Figure 1 a). The reactivity of the antisera against the 20.5 kDa protein was determined by Western blot experiment. The purified protein was electrophoresed in SDS-PAGE gel and the protein separated in the gel was immunoblotted onto nitrocellulose membrane. The blotted membrane was subsequently stained and a single band corresponding to the 20.5 kDa protein was observed (Figure 1 b).

The distribution of the protein in the seeds was analysed by immunolocalization experiments. The developing seeds of *S. bicolor* variety B48826 were used for the study. When sections were stained with antibodies and counter-stained with safranin, prominent staining was observed in the tissues surrounding the embryo, suggesting the presence of the protein in the embryonic tissues (Figure 1 c).

In addition to physical barriers such as lignified testae or pericarp, germinating seeds may utilize physiological strategies to counter microbial invasion, as the



**Figure 1.** *a*, Immunodiffusion assay of polyclonal antisera raised against 20.5 kDa protein. P, Purified 20.5 kDa antifungal protein; AN, Polyclonal antisera raised against the protein; 1 : 1, 1 : 2, 1 : 3, 1 : 4, Dilutions of antisera made with phosphate buffered saline. *b*, Western blot of the purified protein separated in 10% SDS-PAGE showing band corresponding to 20.5 kDa antifungal protein. *c*, Immunolocalization of the 20 kDa purified protein in developing seeds of *Sorghum bicolor*. EA, Embryonic axis; S, Scutellum.

germinating seed contains carbohydrates, proteins and lipid reserves which are attractive targets for microorganisms present in the surrounding soil. The localized expression of defence proteins in the embryonic tissues might constitute an effective barrier to microbial entry into the seed. Presence of proteins in the embryonic region with antifungal activity has been reported previously in maize<sup>5</sup>, tomato<sup>3</sup> and tobacco<sup>22</sup>. Further, it was reported that in maize the resistance against the aflatoxigenic *A. flavus* was associated with the living embryo<sup>23</sup>. Localization of two antifungal proteins, ribosome-inactivating proteins and zeamatin, was also reported in the embryonic tissues of maize. Western blot analyses revealed that RIP-like protein was present at higher levels in the endosperm tissues while zeamatin-like protein was more concentrated in the embryonic tissues<sup>24</sup>.

Studies using two barley aleurone-specific promoters from genes encoding lipid transfer protein and chitinase which were transcriptionally fused with *β*-glucuronidase gene (*GUS*) in transgenic rice revealed the expression of *GUS* fused with chitinase promoter in aleurone layer while expression of *GUS* fused with lipid transfer protein promoter was observed in embryo tissues, revealing the expression of these defence proteins in the embryonic tissues of rice<sup>25</sup>. Reports are available on the presence of antifungal proteins in endosperm tissues of *Sorghum*<sup>12</sup>. Three proteins of molecular weight 18, 26 and 30 kDa were purified and their effect on hyphae of grain mold pathogens like *Fusarium moniliforme*, *F. oxysporum*, *F. spemitectum*, *Alternaria alternata*, *Curvularia lunata*, *Dreschslera rostrata* and *D. longirostrata* was determined. The 18 kDa protein was deter-

mined to be a cell-wall acting protein, while the 26 and 30 kDa proteins caused lysis of the cell wall<sup>17</sup>. Four uncharacterized proteins with molecular weight 20.5, 16.3, 13.9 and 12.2 kDa were purified from the seeds of *S. bicolor* showing high toxicity to aflatoxigenic *A. flavus*<sup>13</sup>. The present study demonstrates the presence of the 20.5 kDa antifungal protein in the embryonic tissues of sorghum seed. Earlier studies have shown the presence of antifungal proteins in the endosperm region of sorghum seeds<sup>12</sup>. The localized expression of different classes of antifungal proteins in various tissues of the seed might constitute an effective barrier against microbial infection.

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MODHUMITA GHOSH<sup>†,\*</sup>  
KANDASAMY ULAGANATHAN<sup>#</sup>

<sup>†</sup>*Institute of Forest Genetics and Tree Breeding, Forest Campus, R.S. Puram,*

*Coimbatore 641 002, India*

<sup>#</sup>*Centre for Plant Molecular Biology, Department of Genetics, Osmania University,*

*Hyderabad 500 007, India*

\*e-mail: ghoshm@ifgtb.res.in

## *Dodonaea angustifolia* – a potential biopesticide against *Helicoverpa armigera*

*Dodonaea*, a common hop bush and a perennial shrub belonging to Sapindaceae is known for its folk remedies. It is distributed from the coast to 2000 m elevated terrain. It is the most aggressive colonizer on disturbed ground, even in rocky gravel or limestone. *D. angustifolia* L.f., predominantly present in India, has a wide range of therapeutic applications since ancient times against pneumonia and other pulmonary diseases including tuberculosis. A decoction of the plant or the wood is used as a purgative in fever and the young twigs are used as tonic<sup>1</sup>.

Tribals of Kolli Hills, Tamil Nadu, use *Dodonaea* as green manure for rice crop along with other plants and have found that the crops show resistance to many pests. Besides, it is observed that *Dodonaea* by itself is free from any pests with the presence of an array of secondary metabolites like catechol, tannins, quinines, saponins, flavones, alkaloids, terpenoids, resins, diterpenoids, phenols, coumarins and essential oil<sup>2</sup>.

*Helicoverpa armigera*, a lepidopteran, commonly known as American boll worm, afflicts nearly 67 cultivated species; the most affected ones are cotton, bhendi (okra), gram species, sunflower and tomato. Of the various strategies adopted to control this pest, no single treatment was successful. Perhaps, repeated application of synthetic pesticides developed resistance in these pests. Loss of control due to the development of multi-resistant

strains has been reported in many crops<sup>3</sup>.

The environmental hazards posed by synthetic pesticides provide an impetus for investigations into some ecofriendly and biorational alternatives. A critical literature survey reveals that *Dodonaea* has not been studied for its pesticidal character, except against cotton leaf worm, *Spodoptera littoralis*<sup>4</sup>. Hence, we aimed to explore the possible biopesticidal activity of this plant to combat the devastating pest *H. armigera*.

The leaves of *D. angustifolia* from Kolli Hills, Tamil Nadu were collected, shade-dried and powdered. Then they were extracted successively using non-polar to polar solvents, viz. hexane, petroleum ether, chloroform and acetone. Aqueous extraction was also done. The plant material was soaked in each solvent for 24 h at 30 ± 2°C, filtered, and to the residue the same solvent was added. This procedure was repeated thrice to obtain maximum extractables. All the filtrates were pooled and evaporated under vacuum in a rotary evaporator<sup>5</sup>. The crude extracts were weighed to measure the yield and then used in desired concentrations for bioassay.

*H. armigera* larvae collected from redgram field were reared in semi-synthetic diet<sup>6</sup>. Each extract at 10% conc was fed to the adult moths and the longevity, fecundity and hatchability were checked. Solvent control (10%) and 10% sugar solution (normal control) were also maintained. Five pairs of adults were released

into the mud pot and kept in the dark. Adult feed was changed daily and the whole set-up was maintained at 22 ± 2°C with 70–75% RH. Triplicates were maintained for each treatment and the data were analysed statistically using Agres package.

Crude concentrate (40 g) of hexane extract was dissolved in hexane (30 ml) and fractionated through a silica-gel column, using solvent combinations of hexane/ethyl acetate, benzene/acetone and petroleum ether/acetone. Totally, 12 fractions were obtained. The eluted fractions were tested against adult moths as mentioned earlier, with their respective controls.

The powdered material of hexane extract (10 g) was eluted in a silica gel column (5 cm × 50 cm), with different solvent combinations, further purified by preparative TLC (on silica gel 60 F<sub>254</sub> gel-coated glass sheets). The purified fraction was subjected to reversed-phase HPLC (on bondapak column with flow rate of 1.5 ml/min and pressure up to 300 psi) using MeOH-H<sub>2</sub>O (9 : 1) as eluent to obtain the pure sample. The single fraction which eluted at 2.9 min was evaporated to dryness and subjected to FTIR (Fourier Transform Infrared). Infra-red spectral data were measured on Perkin-Elmer 1600 series FTIR Spectrometer (Nujol, KBr disks). To determine the molecular weight, the sample was subjected to EIMS (Electro Impact Mass Spectrometry) and ESIMS (Electro