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ORIGINAL ARTICLE

Efficacy of seed defense proteins in biofortified pearl millet lines against blast and downy mildew

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Abstract

Pearl millet is a nutritious and climate-resilient dryland cereal crop. The present study was conducted to identify the defense proteins in seed extrudes of 25 biofortified (Fe and Zn) inbred lines, and investigate their efficacy against blast (isolates Pg 45, Pg 138, and Pg 186) and downy mildew (isolates Sg 409, Sg 445, and Sg 576) diseases. The study revealed the presence of cysteine protease inhibitors (4.3–58.5 units/mg) as well as pathogenesis-related (PR) hydrolases including chitinases (5.3–16.3 units/ml), β-1,3-glucanases (7.3–32.9 units/mg), and cellulases (0.19–4.11 units/mg) in the test lines. The activity levels of cystatins and PR hydrolases in the pearl millet lines were in relative consistency with the resistance levels observed in the greenhouse screenings against blast and downy mildew diseases. Furthermore, antifungal screenings of seed proteins against blast pathogen exhibited a significant reduction in radial growth of Pg 138 (57%) followed by Pg 186 (13%) and Pg 45 (10%). Spectrophotometric assays (A_{595}) exhibited significant retardation in spore germination and initial growth (48 h) of Pg 45 (53.8–87.3%) followed by Pg 186 (19.2–61.3%) and Pg 138 (1.5–36.7%). Furthermore, seed proteins of biofortified lines efficiently reduced the downy mildew disease incidence in greenhouse screenings by seed treatments of susceptible pearl millet lines ICMP 451 (0.6–36% against Sg 409; 32–61% against Sg 576) and 7042R (14–80% against Sg 445). The results of this study will provide insight into the biochemical basis of resistance in pearl millet against foliar blast and downy mildew diseases, and to exploit novel strategies for breeding for disease resistance.

Keywords Blast · Cellulases · Chitinases · Cysteine protease inhibitors · Downy mildew · Glucanases

Introduction

Pearl millet (*Pennisetum glaucum*) is one of the important cereals in the dryland tropical regions of the world, and is widely cultivated in Asia, Africa, and the Americas. It is grown on 31 million ha worldwide primarily for grain production but also valued for fodder, the nutritional importance of which has increased in recent years (ICRISAT 2016). This millet possesses a great yield as well as nutritional advantages, short growing seasons (~ 3 months), economical production cost, and high resilience to extreme abiotic conditions (Jukanti et al. 2016). It is nutritionally rich in protein, dietary fiber, essential amino acids, balanced fatty acids, as well as micronutrients (Rai et al. 2008). The versatile nutraceutical properties due to the presence of unique profiles of phytochemicals as well as antioxidants make pearl millet a major contributor to food and nutritional security of the rural poor in the semi-arid areas of India and Africa (Himanshu et al. 2018). The nutritional (Fe and Zn) content of pearl millet has been further enriched through biofortification breeding at ICRISAT (Rai et al. 2012; Govindaraj et al. 2019a, b). Such nutrient fortification in staple food is one of the sustainable approaches to combat micronutrient malnutrition in developing countries. Nonetheless, the integration of adequate levels of disease resistance is pre-eminent to attain sustainability of fortified nutritional traits in elite cultivars and also to compete with commercial cultivars.

Being a rainy season crop, pearl millet encounters a number of diseases that cause low yield and substantial economic

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loss. This leads to reduced yield potential invariably at the farm level. Among fungal and pseudo-fungal diseases, downy mildew (caused by *Sclerospora graminicola*) and recently emerged blast (caused by *Magnaporthe grisea*, anamorph: *Pyricularia grisea*) are the persistent threats to pearl millet production creating a negative impact on the economy of pearl millet growers (Sharma et al. 2011, 2013). These diseases become more severe during humid weather conditions, particularly in dense plant stands. The usage of synthetic fungicides as an instant preventive strategy is potentially harmful to the environment as well as other non-target organisms, particularly in higher tropic levels of the food chain due to magnified accumulations. In view of this, host plant resistance is one of the cost-effective and environment-friendly approaches for sustainable genetic enhancement for biotic stress tolerance and considered as a key component of the IPM strategy. Hence, identification of resistance sources and sustainable genetic enhancement of disease resistance in the cultivars has always been a major thrust area of pearl millet breeding.

Seeds contain several defense proteins and bioactive compounds such as ribosome-inactivating proteins, lipid transfer proteins, non-protein amino acids (β/γ aminobutyric acid-BABA, GABA), protease inhibitors (PIs), glucanases, cellulases, and chitinases which accumulate in storage vacuoles constitutively and also induced in plant vegetative organs upon biotic stress (Shewry et al. 1995). β-1,3-Glucanases and cellulases are pathogenesis-related (PR-2) hydrolases that, respectively, hydrolyze the glucan and cellulose structural polymers of fungal and oomycete pathogens. While chitinases (PR-3) disintegrate the structural units (β-1,4-*N*-acetyl-D-glucosamine) of chitin in fungal cell wall as well as the cuticle, and gut peritrophic membrane of insects (Iqbal and Anwar 2019). Thus, the PR hydrolases play a prime role in plant defense by destabilizing the structural polymers and rendering the pathogen much vulnerable to other plant defense responses. Also, the released oligosaccharides from the pathogen cell walls act as strong elicitors to induce plant secondary defense response (Ebel and Cosio 1994; Kombrink et al. 2001; Nandini et al. 2017). Hence, tapping these PR hydrolases is a straight forward strategy of biotic stress management as they are involved in providing resistance to both insect pests and pathogens.

PIs (PR-6) are storage proteins that act as a natural phytochemical defense by inactivating the proteases responsible for the growth and survival of fungal pathogens as well as insect pests. As antifungal compounds, they inhibit mycelial extracellular proteases which play a pivotal role in nutrient acquisition from the host plant (Silva et al. 2013; Feldman et al. 2014). While as pesticidal compounds, they irreversibly inactivate larval digestive proteases, in turn, generate critical amino acid deficiency in various metabolic processes, and eventually lead to developmental abnormalities and mortality of pests (Swathi et al. 2016; Lokya et al. 2020). Furthermore, identification of these proteins along with other defense molecules serves as biochemical markers in the differentiation of resistant and susceptible lines, and may also be helpful in resistance breeding (Kini et al. 2000; Feldman et al. 2014). Considering the importance of defense proteins in disease resistance, the present study was aimed to characterize the seed proteins extruded from 25 biofortified inbred lines of pearl millet for the presence of PIs, β-1,3-glucanases, chitinases, and cellulases, and correlate their expression levels with the blast and downy mildew disease resistance of the respective lines. Furthermore, the study was extended to examine in vitro antifungal activity of these seed proteins against three isolates of *M. grisea* (Pg 45, Pg 138, and Pg 186) by radial growth and spore germination inhibition assays as well as to assess the efficacy of seed proteins in reducing the downy mildew disease incidence against *S. graminicola* isolates Sg 409, Sg 445, and Sg 576 by seed treatments of susceptible lines.

Materials and methods

Bovine pancreatic trypsin, bovine pancreatic chymotrypsin, papain from papaya tree latex, and bromelain from pineapple stem were procured from Sisco Research Laboratory (Mumbai, India). Bovine serum albumin (BSA), ficin (fig tree latex), *N*-*α*-benzoyl-DL-arginine-*p*-nitroanilide (BA*p*NA), *N*-succinyl–Ala–Ala–Pro–Phe *p*-nitroanilide (SAA*p*NA), pGlu–Phe–Leu–*p*-nitroanilide (*p*FLNA), *N*-acetyl-D-glucosamine, laminarin, crab shell chitin, and dithiotheitol (DTT) were purchased from Sigma (St. Louis, MO). Glucose estimation kit procured from Kamineni Life Sciences, Hyderabad. X-ray films were purchased from Carestream Health India Pvt Ltd. (Mumbai). All other chemicals and reagents used were of analytical grade.

Genetic material selection

Twenty-five biofortified pearl millet inbred lines consisting of 10 B-lines ICMB 1604, ICMB 100267, ICMB 101439, ICMB 101088, ICMB 1608, ICMB 100376, ICMB 97222, ICMB 1502, ICMB 1507, ICMB 100662 and 15 R-lines ICMR 100014, ICMR 1505, ICMR 1504, ICMR 102088, ICMR 100101, ICMR 100016, ICMR 100244, ICMR 100004, ICMR 100025, ICMR 13777, ICMR 101012, ICMR 100023, ICMR 100040, ICMR 100098, and ICMR 100021 with higher but diversified micronutrient content (Fe 55–164 ppm; Zn 31–79 ppm) and varied maturity groups of early to late (41–59 days to flower) were selected (Supplementary Table 1). All these lines were biofortified using classical recombinant breeding approaches (Govindaraj et al. 2019a). Briefly, biofortification was achieved by crossing

among the high Fe/Zn lines and later to the elite lines followed by pedigree breeding to fix the line with high Fe/Zn in agronomically superior backgrounds at F_6 and later stages. These micronutrients are highly heritable and predominantly controlled by additive genes, and therefore, it is highly feasible to breed biofortified lines in pearl millet. Pearl millet biofortification breeding approaches are well described elsewhere (Rai et al. 2012; Govindaraj et al. 2013, 2019b).

Pathotype-isolate selection

The highly virulent pathotype-isolates of *M. grisea* collected from pearl millet fields from Patancheru, Telangana (Pg 45), Jaipur, Rajasthan (Pg 138), and Aurangabad, Maharashtra (Pg 186) in India, and being maintained in Cereals Pathology, ICRISAT were used in the present study. Similarly, *S. graminicola* pathotype-isolates collected from Patancheru, Telangana (Sg 409), Banaskantha, Gujarat (Sg 445), and Jodhpur, Rajasthan (Sg 576), and being maintained through asexual generations under controlled greenhouse conditions in Cereals Pathology, ICRISAT were selected.

Greenhouse screening against foliar blast

Twenty-five pearl millet inbred lines were screened for foliar blast resistance against three pathotype-isolates Pg 45, Pg 138, and Pg 186 along with susceptible (ICMB 95444) check. Seeds of these lines were sown in 15-cm diameter pots filled with sterilized soil-sand-FYM (farmyard manure) in 2:1:1 ratio and maintained at 30 ± 1 °C in a greenhouse. Twelve-day old seedlings were spray inoculated with an aqueous spore suspension (1×10^5 spores/ml) of each pathotype-isolate of *M. grisea* (Pg 45, Pg 138, Pg 186) independently followed by converging the seedlings with polythene bags for 48 h to avoid cross-contamination and incubation at 25 °C. The pots were then exposed to high humidity under misting for 4 days in greenhouse chambers. Foliar blast severity on each entry was recorded after 6 days of inoculation using 1–9 progressive scale (Sharma et al. 2013). The experiment was conducted twice in a completely randomized design (CRD) with two replicates (one pot per replicate) and 10 seedlings were maintained per replicate.

Greenhouse screening against downy mildew

The 25 pearl millet inbred lines were screened for resistance against three pathotype-isolates of *S. graminicola* (Sg 409, Sg 445, and Sg 576) under greenhouse conditions (Thakur et al. 2009). The inoculum of each isolate was harvested from the infected sporulating leaves in sterilized ice-cold distilled water separately and adjusted spore concentration to 5×10^5 sporangia/ml. The 48 h old seedlings were spray inoculated with sporangia of each isolate independently, protected with polythene sheets to avoid cross-contamination, and incubated at 20 °C for 24 h. Subsequently, pots were shifted to greenhouse benches and maintained for 2 weeks under standard conditions (25 ± 2 °C, > 95% RH). Disease incidence against each isolate was recorded after 14 days of inoculation as a percentage of the infected seedlings. The reliability of the disease screen was assessed by incorporating the susceptible check in the study. The experiment was conducted twice in a completely randomized design (CRD) with two replicates (two pots per replicate) and 35–40 seedlings were maintained per pot.

Fe and Zn estimation

The grain Fe and Zn densities were analyzed using energy-dispersive X-ray fluorescence spectrometry (XRF) that was calibrated based on inductively coupled plasma optical emission spectrometer (ICP-OES) values (Govindaraj et al. 2016). Scans were conducted using A1 sample cups of 30 mm diameter, 36 mm depth, and > 20 g of grain weight capacity which is accompanied by polypropylene inner cups. The grain samples were loaded into cups with even distribution and scanned for Fe and Zn densities.

Seed protein extraction

The seed proteins were extracted from dry mature seeds of selected pearl millet lines according to the procedure described by Prasad et al. (2009). The fine powder prepared from seeds was depigmented and defatted with three washes of acetone and hexane, respectively. Seed proteins were extracted into 1:6 (w/v) volumes of 50-mM Tris HCl, pH 8.0 containing 1% polyvinylpyrrolidone under cold (4 °C) conditions for overnight. The crude extracts were centrifuged at 10,000 rpm for 20 min at 4 °C and obtained clear supernatants were extruded using 0.2-µm syringe filters (Techno plastic products limited, Switzerland) under sterile conditions and proceeded for further studies as crude seed proteins. The total protein content was determined by the micro-Kjeldahl method (Kjeldahl 1883).

Protease inhibitor assays

Protease inhibitor activity of seed proteins against serine and cysteine proteases was performed spectrophotometrically using synthetic chromogenic substrates. The amount of seed protein (7–35 µg) that yields 40–60% reduction in the respective protease activity was measured colorimetrically (A_{415}) using BA*p*NA for trypsin (Erlanger et al. 1961), SAA*p*NA for chymotrypsin (Mueller and Weder 1989) and pGlu–Phe–Leu–*p*-nitroanilide (pFLNA) for cysteine proteases papain, bromelain, and ficin (Filippova et al. 1984) as substrates. Briefly, an appropriate amount of seed protein in

assay buffer 0.05 M Tris–HCl, and 0.02 M $CaCl_2$ containing trypsin (10 µg) pH 8.2 or chymotrypsin (20 µg) pH 7.8 was incubated for 15 min at 37 °C. In the case of cysteine proteases 0.1 M sodium acetate pH 6.5 containing 8 mM DTT, 4 mM EDTA was used as assay buffer for papain (30 µg) and bromelain (30 µg) and incubated at 60 °C for 5 min. Whereas ficin (1 µg) inhibitory activity was determined using 0.1 M sodium acetate pH 6.5 assay buffer which is devoid of DTT and EDTA. Change in absorbance (A_{415} nm) was monitored for 15 min after the addition of BApNA/SAApNA/pFLNA (1 mM). One inhibition unit is defined as the lowest amount of seed protein that required inhibiting 50% of respective protease activity under optimal assay conditions.

Dot blot assay

The protease inhibitor activity of seed proteins was monitored by the X-ray film contact print method developed by Pichare and Kachole (1994). Gelatin coated X-ray film was spotted with enzyme and seed protein mixture (1:1 v/v) which was pre-incubated at 37 °C for 10 min to allow optimal inhibition of the enzyme active site. Enzyme mixed with respective assay buffer (1:1 v/v) devoid of seed protein was used as control. The spotted film was incubated for 20 min at 37 °C and the extent of gelatin hydrolysis on the X-ray film was monitored as the presence of protease activity by visual blue color change after washing the film under running tap water. The presence of protease inhibitor activity is inversely related to the area of gelatin hydrolysis on X-ray film.

β-1,3-Glucanase assay

The β-1,3-glucanase activity in seed proteins was assayed by measuring the rate of reducing sugar (glucose) generated using laminarin as a substrate (Koga et al. 1988). Briefly, the reaction mixture consisted of 20 µl laminarin (1%) and various volumes (2.5, 10 µl) of seed protein in the assay buffer of 0.05 M sodium acetate pH 5.2 (100 µl). The reaction was carried out at 50 °C for 10 min and resulting glucose was measured enzymatically by the addition of glucose oxidase peroxidase reagent (100 µl) from the glucose estimation kit. The resultant glucose concentration was spectrophotometrically (A_{490}) measured using glucose (0–1 mg/ml) as an internal standard. The activity of β-1,3-glucanase was expressed as units/mg protein.

Chitinase assay

The level of chitinases present in seed proteins was determined colorimetrically by modified Schale's method using colloidal chitin as a substrate (Ferrari et al. 2014). Colloidal chitin was prepared using crab shell chitin flakes according to the method described by Wu et al. (2009). The assay was performed using 1% colloidal chitin (100 µl) in sodium acetate buffer (0.05 M, pH 5.7) mixed with various concentrations of seed proteins and incubated under mild stirring conditions for 1 h at 37 °C. After incubation, the reaction was stopped by addition of 0.5 M sodium hydroxide (100 µl) and centrifuged at 10,000 rpm for 15 min. The collected supernatant (100 µl) was mixed and incubated (65 °C, 10 min) with 300 µl of Schale's reagent (0.5 M sodium carbonate and 0.5 g/l potassium ferricyanide). The released monomeric *N*-acetyl-D-glucosamine (GlcNAc) was monitored spectrophotometrically as a change in absorbance at 420 nm. GlcNAc (0–5 mg/ml) was used as an internal standard and positive control.

Cellulase assay

The cellulase activity present in seed proteins was determined by modified filter paper assay which is recommended by IUPAC (International Union of Pure and Applied Chemistry) using a plate reader (Yu et al. 2016). Whatman filter paper (No. 1) was used as a substrate by cutting into discs (0.5 cm diameter) using a common office hole punch and each disc was accommodated at the bottom of 96-well microplate. Seed proteins (10 µl) mixed with 0.05 M sodium citrate buffer pH 5.0 (200 µl) were incubated in each well at 37 °C with mild stirring conditions for 1 h. Plates were centrifuged at 3000 rpm for 5 min and 100 µl of each supernatant transferred to a fresh plate and mixed with 100 µl of glucose oxidase peroxidase reagent from glucose estimation kit. The release of glucose was monitored colorimetrically (A_{490}) at 37 °C for 10 min using glucose (0–1 mg/ml) as an internal standard. As per the IUPAC standardization, the expected amount of cellulase that releases 80 µg of glucose from filter paper corresponds to 0.37 units. Accordingly, the activity of cellulase was expressed in standard units of filter paper assay (FPA = 0.37/amount of cellulase required to release 80 µg of glucose).

Radial growth inhibition assays of *M. grisea*

The antifungal activity of seed proteins was assayed against mycelial growth of *M. grisea* isolates Pg 45, Pg 138, and Pg 186 (Cole 1994). The study was executed using Petri dishes (90 mm diameter) containing 20 ml of oat meal agar (OMA) medium under aseptic conditions. The sterilized (0.2 µm syringe filters) seed proteins (0.01% w/v) were rapidly incorporated and mixed well with OMA before pouring into plates. After the OMA had cooled down, a peripheral plug (2 mm) picked from the actively growing culture of each isolate was placed at the center of the agar plates. OMA plate devoid of seed proteins served as a control, and considered to compare the reduction in fungal growth of the pathogen isolates in test plates. All the plates were incubated

under standard conditions (25 °C) and maintained until the mycelial growth in the control plate reached from central to the peripheral region (~ 10–13 days). The effect of seed extract of each line on the growth rate of *M. grisea* was assessed after every 24 h by monitoring the growth curves plotted based on colony diameter against time. The experiment was repeated twice each with two replicates.

Inhibition of spore germination and initial growth of *M. grisea*

The antifungal activity of seed proteins was measured quantitatively by spectrophotometric assays using 96-well microtiter plates according to the method reported by Broekaert et al. (1990). Briefly, the assay mixture consisted of 5 µl of seed protein to be assayed, 50 µl of diluted spore suspension, and 145 µl of potato dextrose broth (PDB). Microcultures with sterile water instead of seed protein served as controls. The initial absorbance was measured at 595 nm after allowing the spores to sediment for 30 min at 25 °C using a plate reader. Inhibition in fungal growth was monitored (A_{595} nm) after incubating the plates at 25 °C for 48 h. Inhibition in mycelial growth was calculated according to the equation $[(\Delta C - \Delta T)/\Delta C] \times 100$, where ΔC and ΔT are the corrected absorbance of the control and test samples, respectively. The corrected absorbance is the subtracted value from the final A_{595} (48 h) minus initial A_{595} (30 min).

Efficacy of seed proteins against downy mildew

The seed proteins of 25 inbreds were evaluated for their potential in suppressing downy mildew disease incidence by seed treatments followed by greenhouse screenings (Mythrashree et al. 2013). For this, seeds of pathotype-specific pearl millet susceptible lines (7042R and ICMP 451) were treated with seed protein of each of the 25 inbred lines (4% v/v) for 15 h. Seeds treated with buffer (0.05 M Tris pH 8.0) in which seed proteins were extracted served as control. Treated and untreated seeds were sown in pots (two replications, 35–40 seedlings per replication) and screened against three pathotype-isolates (7042R screened against Sg 445, and ICMP 451 screened against Sg 409 and Sg 576) under greenhouse conditions as described above. A highly susceptible line 7042S was included as a check. Disease incidence (%) was recorded in treated and untreated (control) pots after 2 weeks of inoculation (Thakur et al. 2009).

Statistical data

The analysis of variance for the activity of defense enzymes PI, glucanases, chitinases, and cellulases in the 25 biofortified pearl millet inbred lines, and their efficacy against *M. grisea* and *S. graminicola* was carried out using the GENSTAT statistical package (version138 10.1; Rothamsted Experiment Station, Herpenden, Herts, UK) (Payne 2002). The percent data on inhibition of growth of *M. grisea* isolates and downy mildew incidence caused by *S. graminicola* isolates were transformed using arcsine transformation and analyzed. A Duncan multiple range test at $P \leq 0.05$ was used to compare averages of activities of defense enzymes in the pearl millet inbred lines and their efficacy in percent reduction in the growth of *M. grisea* isolates and downy mildew incidence caused by *S. graminicola* isolates.

Results

Total protein content and Fe and Zn densities

The total protein content, and Fe and Zn densities in the dry seeds of biofortified 25 pearl millet lines are given in Table 1. The protein content in grain samples varied from

Table 1 Total seed protein (%) content, and Fe and Zn densities in biofortified pearl millet inbred lines

S. no.	Genotype/inbred line	Protein (%)	Fe (ppm)	Zn (ppm)
1	ICMB 1604	13.63	77	48
2	ICMB 100267	12.85	85	47
3	ICMB 101439	15.19	164	79
4	ICMB 101088	11.58	83	52
5	ICMB 1608	13.68	108	53
6	ICMB 100376	10.65	76	42
7	ICMB 97222	14.85	74	50
8	ICMB 1502	13.93	92	50
9	ICMB 1507	12.14	92	50
10	ICMB 100662	13.12	79	53
11	ICMR 100014	11.85	60	39
12	ICMR 1505	13.56	86	35
13	ICMR 1504	10.0	101	51
14	ICMR 102088	11.37	110	52
15	ICMR 100101	13.65	69	47
16	ICMR 100016	10.53	64	40
17	ICMR 100244	10.60	83	37
18	ICMR 100004	13.31	75	58
19	ICMR 100025	11.13	67	44
20	ICMR 13777	13.28	92	61
21	ICMR 101012	11.26	99	53
22	ICMR 100023	10.71	55	31
23	ICMR 100040	11.02	113	67
24	ICMR 100098	11.14	113	77
25	ICMR 100021	11.37	57	35
	Min	10	55	31
	Max	15.19	164	79
	LSD ($P = 0.05$)	0.94	13.64	7.05

10 to 15%. The Fe density varied from 55 to 164 ppm with an average of 87 ppm, while Zn density among the 25 pearl millet lines varied between 31 and 79 ppm with an average of 50 ppm.

Greenhouse screening against blast disease

Among tested lines, 13 (4 B, 9 R) lines were resistant to Pg 45 (1–3 score) and eight (4 B, 4 R) were moderately resistant (˃ 3–5 score) as represented in Table 2. In case of Pg 138, none of the test lines was found resistant, whereas 15 (6 B, 9 R) lines were moderately resistant. Likewise, 12 (4 B, 8 R) lines were resistant and 11 (5 B, 6 R) lines were moderately resistant against Pg 186. Altogether, three B-lines ICMB 100267, ICMB 101088, ICMB 1604, and seven R-lines ICMR 100004, ICMR 100021, ICMR 100040, ICMR 100101, ICMR 102088, ICMR 13777, ICMR 1504 exhibited resistance to both Pg 45 and Pg 186. The susceptible check ICMB 95444 recorded a disease score of 7–9.

Greenhouse screening against downy mildew

Among 25 test lines, 14 (3 B and 11 R-lines) lines were resistant to Sg 409 (≤ 10% disease incidence) and three lines (2 B and 1 R) were moderately resistant (˃ 10–20% disease incidence). In case of Sg 445, 11 (2 B and 9 R) lines were resistant, and three (1 B and 2 R) lines were moderately resistant (Table 2). Similarly, 16 (6 B, 10 R) lines were found to be resistant to Sg 576, and one R-line was moderately resistant to this pathotype-isolate. Overall, seven R-lines ICMR 100014, ICMR 1505, ICMR 102088, ICMR 100101, ICMR 100016, ICMR 100025, and ICMR 100023 exhibited resistance against all the three pathotype-isolates Sg 409, Sg 445, and Sg 576, whereas six lines, including four B-lines ICMB 1604, ICMB 100267, ICMB 101439, and ICMB 1502, and two R-lines ICMR 100244 and ICMR 100004 were resistant to any two of the three pathotype-isolates of *S. graminicola*.

Protease inhibitor (PI) activity

The seed proteins were evaluated for the presence of PI activity against two serines (trypsin and chymotrypsin) and three cysteine proteases (papain, bromelain, and ficin). As a result, seed proteins exhibited low-to-moderate inhibition (0–31%) against papain, and no inhibition was observed against trypsin, chymotrypsin, and bromelain (data not shown). Nevertheless, all the tested seed proteins exhibited significant inhibitory activity 25–61% against ficin protease with a range of specific activity 4.3–58.5 units/mg protein (Table 3). The high PI activity was observed in the seeds of ICMR 100101 (58.5 units/mg protein) and ICMR 100040 (56 units/mg protein). Whereas low PI activity was observed in ICMR 100098 (4.3 units/mg protein) and ICMB 1608 (6.3 units/mg protein).

Dot blot assays

The wide variability in the PI activity of seed proteins against ficin protease was further evidenced through dot blot assays using gelatin-coated X-ray film (Fig. 1). The inhibition of gelatin hydrolysis on X-ray film indicates the presence of PI activity in seed proteins. A clear zone showing hydrolysis of gelatin was observed in the control, which consisted of ficin enzyme alone in the buffer and devoid of PIs present in seed proteins. Lines with high PI activity inhibited the gelatin hydrolysis on X-ray film in contrast to lines with low PI activity. Complete inhibition of gelatin hydrolysis was observed with the lines ICMB 1604, ICMB 101088, ICMR 100014, ICMR 1504, ICMR 102088, ICMR 100101, ICMR 100023, and ICMR 100040 having moderate-to-high PI activity ranging from 38 to 58 units/mg protein in spectrophotometric assays. In contrast, absolute gelatin digestion was observed in the presence of seed proteins of ICMB 1608, ICMB 100662, ICMR 100098, and ICMR 100021 in which PI activity ranged between 4.3 and 8.2 units/mg protein.

Determination of β-1,3-glucanase activity

In the present study, β-1,3-glucanase activity levels in seed proteins of test lines ranged between 7.3 and 32.9 units/mg protein (Table 3). The highest activity of this enzyme was observed in ICMR 100101 (32.94 units/mg protein) which is significantly higher than the activity observed in other pearl millet lines. Higher activity of glucanase in ICMR 100101, ICMB 101439 (27.07 units/mg protein), ICMR 100016 (27.89 units/mg protein), and ICMR 100244 (27.42 units/mg protein) is in consistence with the downy mildew as well as foliar blast disease resistance levels observed in the greenhouse screenings (Table 2). Whereas, isolate specific susceptibility was observed in ICMB 100662 (susceptible to all isolates), ICMR 13777 (susceptible to Sg 445, Sg 576 and Pg 138), ICMB 1507 (susceptible to Pg 138, Sg 409 and Sg 445), ICMR 100098 (resistant to only Sg 576), and ICMR 100021 (susceptible to Pg 138, Sg 409, Sg 445 and Sg 576) with low levels of β-1,3-glucanase activity (7.75–14.91 units/mg protein). ICMB 1502 also had low β-1,3-glucanase activity, and was found to be moderately resistant to blast and susceptible to Sg 445.

Determination of chitinase activity

Chitinases, which are known to play a prime role in antifungal defense, were found to vary between 5.27 and 16.33 units/ml among seed proteins of inbred lines (Table 3).

Table 2 Greenhouse screening of 25 biofortified pearl millet inbred lines for resistance against foliar blast and downy mildew

S. no.	Genotype/inbred line	*Magnaporthe grisea* pathotype-isolate						*Sclerospora graminicola* pathotype-isolate					
		Pg 45 (Patancheru)		Pg 138 (Jaipur)		Pg 186 (Aurangabad)		Sg 409 (Patancheru)		Sg 445 (Banaskantha)		Sg 576 (Jodhpur)	
		Blast score[c]	Reaction	Blast score[c]	Reaction	Blast score[c]	Reaction	Incidence (%)	Reaction	Incidence (%)	Reaction	Incidence (%)	Reaction
1	ICMB 1604	3	R	5	MR	2	R	9	R	74	S	0	R
2	ICMB 100267	3	R	6	S	2	R	37	S	0	R	0	R
3	ICMB 101439	4	MR	4	MR	3	R	0	R	32	S	0	R
4	ICMB 101088	3	R	4	MR	3	R	18	MR	78	S	25	S
5	ICMB 1608	6	S	6	S	5	MR	14	MR	3	R	21	S
6	ICMB 100376	3	R	4	MR	4	MR	46	S	14	MR	76	S
7	ICMB 97222	4	MR	5	MR	4	MR	30	S	51	S	0	R
8	ICMB 1502	4	MR	4	MR	4	MR	2	R	100	S	0	R
9	ICMB 1507	4	MR	6	S	5	MR	28	S	97	S	8	R
10	ICMB 100662	8	S	6	S	7	S	99	S	99	S	69	S
11	ICMR 100014	4	MR	4	MR	3	R	0	R	3	R	0	R
12	ICMR 1505	4	MR	6	S	5	MR	1	R	3	R	6	R
13	ICMR 1504	3	R	4	MR	3	R	4	R	21	S	54	S
14	ICMR 102088	2	R	4	MR	2	R	0	R	4	R	0	R
15	ICMR 100101	2	R	4	MR	3	R	0	R	4	R	0	R
16	ICMR 100016	5	MR	4	MR	4	MR	3	R	1	R	0	R
17	ICMR 100244	6	S	4	MR	4	MR	12	MR	3	R	0	R
18	ICMR 100004	3	R	6	S	3	R	41	S	4	R	0	R
19	ICMR 100025	3	R	4	MR	4	MR	1	R	1	R	5	R
20	ICMR 13777	3	R	7	S	3	R	2	R	66	S	58	S
21	ICMR 101012	3	R	4	MR	5	MR	0	R	12	MR	33	S
22	ICMR 100023	4	MR	6	S	4	MR	2	R	0	R	0	R
23	ICMR 100040	2	R	4	MR	3	R	0	R	16	MR	15	MR
24	ICMR 100098	7	S	7	S	8	S	92	S	100	S	0	R
25	ICMR 100021	2	R	7	S	2	R	98	S	100	S	95	S
	[a]ICMB 95444	8	S	7	S	9	S	–	–	–	–	–	–
	[b]7042S	–	–	–	–	–	–	97	S	100	S	97	S
	LSD ($P=0.05$)	1.27		0.58		1.21		1.56		1.93		2.24	

Disease reaction: *R* resistant (≤ 3 blast score; ≤ 10% downy mildew incidence), *MR* moderately resistant (> 3–5 blast score; > 10–20% downy mildew incidence), *S* susceptible (> 5 blast score; > 20% downy mildew incidence)

[a]Susceptible check for blast

[b]Susceptible check for downy mildew

[c]Blast score on 1–9 scale

Table 3 Protease inhibitor (against ficin), and pathogenesis-related (PR) hydrolases, glucanases, chitinases, and cellulases activities in seed proteins of biofortified pearl millet inbred lines

S. no.	Genotype/inbred line	Protease inhibitors[#]	Glucanases[c]	Chitinases[&]	Cellulases*
1	ICMB 1604	53.63^{s}	22.50^{fgh}	12.86^{ijk}	0.97^{ef}
2	ICMB 100267	36.18^{m}	25.00^{ghij}	13.50^{jk}	0.78^{cde}
3	ICMB 101439	45.50^{q}	27.07^{ij}	16.33^{l}	1.29^{hi}
4	ICMB 101088	38.30^{n}	25.12^{ghij}	11.91^{hij}	4.11^{o}
5	ICMB 1608	6.31^{b}	18.13^{de}	7.38^{bcd}	0.83^{cde}
6	ICMB 100376	43.73^{p}	25.65^{ghij}	9.15^{def}	1.46^{ijk}
7	ICMB 97222	26.83^{i}	24.70^{ghij}	10.43^{fgh}	0.77^{cd}
8	ICMB 1502	10.20^{d}	7.32^{a}	6.93^{abc}	0.48^{b}
9	ICMB 1507	11.60^{e}	12.34^{bc}	7.50^{bcd}	0.64^{bc}
10	ICMB 100662	8.21^{c}	7.75^{a}	8.52^{cde}	0.29^{a}
11	ICMR 100014	41.02^{o}	25.13^{ghij}	12.03^{hij}	2.12^{m}
12	ICMR 1505	32.78^{l}	20.34^{ef}	10.63^{fgh}	1.80^{l}
13	ICMR 1504	45.69^{q}	21.78^{efg}	14.35^{k}	1.52^{jk}
14	ICMR 102088	52.10^{r}	26.33^{hij}	11.02^{gh}	1.60^{k}
15	ICMR 100101	58.45^{u}	32.94^{k}	9.70^{efg}	3.01^{n}
16	ICMR 100016	13.07^{f}	27.89^{j}	7.64^{bcd}	1.47^{ijk}
17	ICMR 100244	22.97^{h}	27.42^{j}	7.03^{bc}	1.07^{fg}
18	ICMR 100004	31.55^{k}	14.18^{c}	11.70^{hi}	0.50^{b}
19	ICMR 100025	33.02^{l}	22.80^{fghi}	11.00^{gh}	1.34^{hij}
20	ICMR 13777	16.79^{g}	8.44^{a}	9.00^{def}	0.25^{a}
21	ICMR 101012	28.67^{j}	27.00^{ij}	10.71^{fgh}	0.86^{de}
22	ICMR 100023	39.02^{n}	22.90^{fghi}	8.34^{bcde}	1.20^{gh}
23	ICMR 100040	56.02^{t}	24.39^{fghij}	10.36^{fgh}	0.75^{cd}
24	ICMR 100098	4.31^{a}	9.08^{ab}	6.65^{ab}	0.19^{a}
25	ICMR 100021	6.81^{b}	14.90^{cd}	5.27^{a}	0.21^{a}
	Mean	30.51	20.84	10.0	1.18
	LSD ($P=0.05$)	1.032	3.64	1.58	0.17

Values followed by the same small letter within a column are not significantly different ($P=0.05$) (Duncan's multiple range test)

[#]Protease inhibitor activity (inhibition units/mg protein)

[$]β-1,3-Glucanase activity (activity units/mg protein)

[&]Chitinase activity (units/ml)

*Cellulase activity (FPA units/mg protein)

Among B-lines, ICMB 101439 exhibited the highest chitinase activity of 16.33 units/ml, whereas ICMR 1504 exhibited maximum activity of 14.35 units/ml among R-lines. The level of chitinase activity was in accordance with leaf blast resistance observed in the greenhouse screenings.

Determination of cellulase activity

Cellulase activity level among inbreds was found to vary between 0.19 and 4.11 units/mg protein (Table 3). Prominent levels of the activity were observed in ICMB 101088 (4.11 units/mg protein) and ICMR 100101 (3.01 units/mg protein). Low level of cellulase activity was observed in ICMR 100098 (0.19 units/mg protein), ICMR 100021 (0.21 units/mg protein), ICMR 13777 (0.25 units/mg protein), and ICMB 100662 (0.29 units/mg protein). The level of cellulase activity is in apparent consistency with the downy mildew resistance observed in greenhouse screenings.

Mycelial growth inhibition assays of *M. grisea*

Seed proteins of inbreds exhibited significant inhibition of 9–57% in mycelial radial growth of Pg 138. A prominent inhibition was observed by seed proteins of 12 lines including ICMR 100014, ICMR 1505, ICMR 102088, ICMR 100101, ICMR 100016, ICMR 100244, ICMR 100004, ICMR 100025, ICMR 13777, ICMR 101012, ICMR 100023, and ICMR 100040 when compared with control (Supplementary Fig. 1). Maximum inhibition (%) was observed with ICMR 100025 (57%) and ICMR 13777 (53%) which is represented in growth curves (Fig. 2a–c), while a relatively low level of inhibition was observed for Pg 45 (10% with

Fig. 1 Representation of ficin protease inhibitor (PI) activity of seed proteins using X-ray dot blot assays. The unhydrolyzed gelatin in the spotted area indicates the presence of PI activity

ICMB 101439) and Pg 186 (13% with ICMR 100023; data not shown).

The effect of seed proteins on spore germination and initial mycelial growth (48 h) of respective pathotype-isolates (Pg 45, Pg 138, and Pg 186) in the presence of seed protein of each inbred line was quantitatively (A_{595}) examined (Table 4). Significant differences in the reduction of initial mycelial growth of *M. grisea* isolates were observed with the seed proteins of inbred lines which was found to be more prominent against Pg 45 (53.8–87.3%) followed by Pg 186 (19.2–61.3%) and Pg 138 (1.5–36.7%). Maximum reduction in the growth of *M. grisea* isolates Pg 45, Pg 138, and Pg 186 was observed with the seed proteins of ICMR 13777, ICMB 101439, and ICMR 100025, respectively. The seed proteins of ICMR 100021 were least effective against Pg 45 and Pg 186, whereas ICMR 100014 was least effective against Pg 138.

Efficacy of seed proteins against downy mildew incidence

Significant reduction in the downy mildew incidence in the pathotype-specific susceptible lines, ICMP 451 and 7042R, was observed against all the three *S. graminicola* isolates following seed treatment with the seed proteins of biofortified inbred lines. The downy mildew incidence in the seedlings of ICMP 451 raised from seed treated with the seed proteins ranged between 35.5 and 62.5% when screened against Sg 576 which corresponds to 61.3–32.0% reduction over control (untreated seed) (Table 5). Similarly, downy mildew incidence in ICMP 451 against Sg 409 ranged between 38.8 and 60.3% with the seed treatment which corresponds to 36.0–0.6% reduction over control. A significantly low disease incidence against Sg 409 was observed following seed treatments with the seed proteins of ICMR 102088, ICMR 100016, ICMR 101012, ICMB 97222, and ICMB 1507. Whereas in the case of Sg 576, prominent disease reduction was observed with treatments of seed proteins from ICMB 100376, ICMR 102088, ICMR 100025, and ICMR 100098 lines. Significant reduction in downy mildew incidence was also observed in 7042R against Sg 445 with the seed treatment and it ranged between 8.3 (ICMB 1608) and 36.1% (ICMB 100662) which corresponds to 80.3–14.4% reduction over untreated control. A notable decrease in disease incidence was observed when 7042R seeds were treated with the seed proteins of ICMB 1608, ICMR 100004, ICMB 100267, and ICMR 1504.

Discussion

Seed defense proteins such as pathogenesis-related (PR) proteins, defensins, thionins, lectins, and many others play an important role in plant resistance. One of the prominent features of defense proteins is the antifungal activity, which is

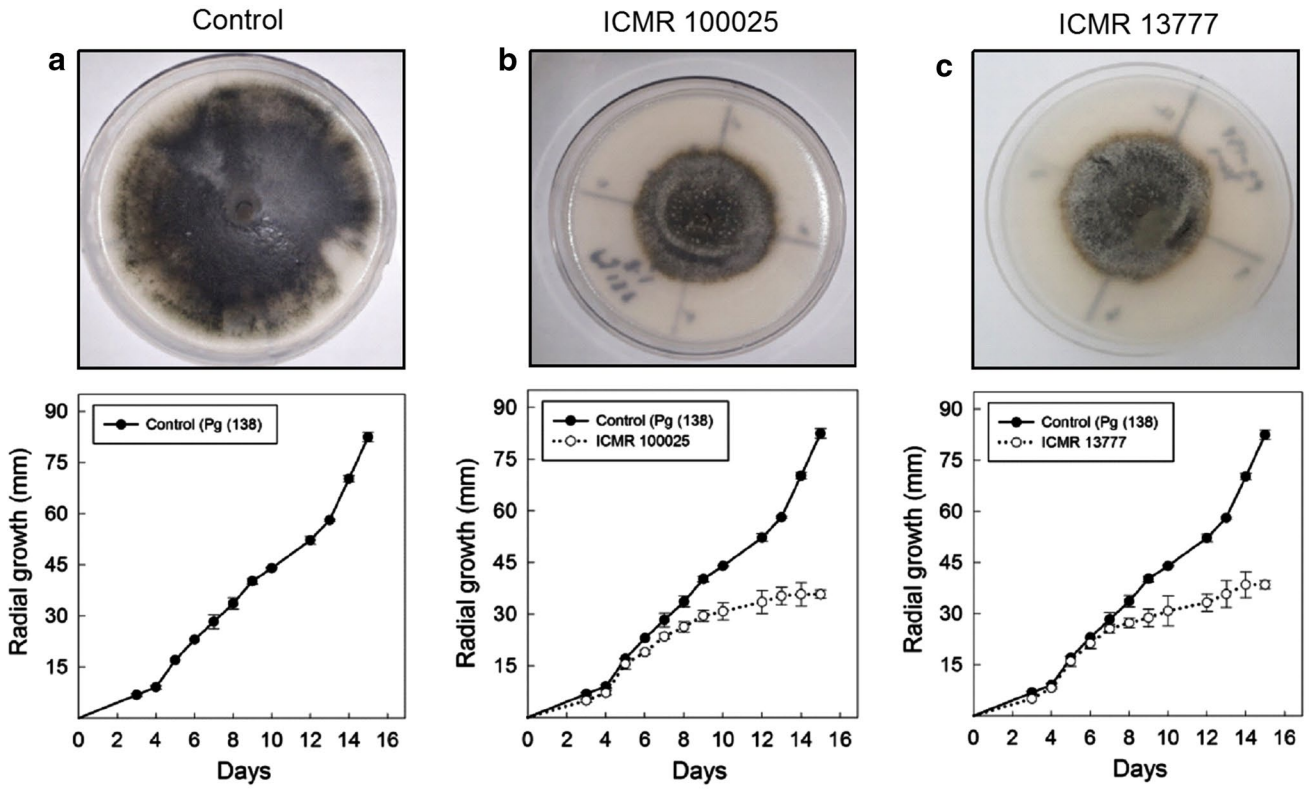

Fig. 2 Antifungal activity of pearl millet seed proteins against *M. grisea*. The radial growth inhibition of Pg 138 fungal zones and respective growth curves in **a** control (OMA without seed proteins) and two representative seed protein samples, **b** ICMR 100025 and **c** ICMR 13777 are illustrated

generally accounted for their protease inhibitor, hydrolytic, and membrane permeability actions (Yan et al. 2015). Also, the evaluation of seed defense proteins as biochemical markers is one of the reliable options as they are largely independent of environmental fluctuations (Iqbal et al. 2005). In the present study, efforts were made to correlate the levels of seed defense proteins cystatins and PR hydrolases (β-1,3-glucanases, chitinases, and cellulases) in the 25 Fe and Zn biofortified pearl millet inbred lines with disease resistance against blast (Pg 45, Pg 138, and Pg 186) and downy mildew (Sg 409, Sg 445, and Sg 576) observed in these lines in the greenhouse screens.

The eminent existence of cysteine PIs against ficin protease with 25–61% inhibition and 4–58 units/mg protein of the specific activity was evidenced by spectrophotometric as well as X-ray dot blot assays (Fig. 1 and Table 3). The secreted proteases from pathogenic fungi play a pivotal role in invasion and nutrient acquisition from the host plant (Niño et al. 2014; Muszewska et al. 2017). The diverse classes of extracellular proteases released into media from rice blast pathogen *M. oryzae* are well characterized by Machin et al. (2008). Particularly, the prominent role of cysteine proteases in pathogenesis is evidenced in leaf pathogen (*Pyrenopeziza brassicae*) causing damage to plants of Cruciferae family. A non-pathogenic mutant of *P. brassicae* was unable to produce extracellular cysteine proteases, whereas recovery of pathogenesis in these mutants was achieved with the recovery of their ability to produce cysteine proteases (Ball et al. 1991). In the present study, the activity levels of cystatins were in a positive correlation with the greenhouse disease resistance levels against blast (ρ 0.43), downy mildew (ρ 0.12), and both blast and downy mildew together (ρ 0.44). The comparative alliance of cystatins towards blast resistance could be due to inhibition of extracellular proteases and attenuation of chitin synthase activity (Joshi et al. 1998; Niño et al. 2014). Similarly, the antifungal role of cystatins in host plant resistance is well underlined in barley, chestnut, sugarcane, taro, tomato, and wheat (Pernas et al. 1999; Siqueira-Junior et al. 2002; Soares-Costa et al. 2002; Yang and Yeh 2005; Christova et al. 2006).

Seed proteins were further evaluated for the presence of β-1,3-glucanases (7.3–32.9 units/mg protein), chitinases (5–16 units/ml) and cellulases (0.19–4.11 units/mg protein) (Table 3) as they are important determinants in antifungal resistance (Funnell et al. 2004). The assessed activity levels of PR hydrolases are in positive correlation with the disease resistance levels against blast and downy mildew observed in the greenhouse screens (Tables 2, 3). Interestingly, the

Table 4 Spore germination and initial growth inhibitory activity (A_{595}) of seed proteins of 25 biofortified pearl millet inbred lines against *Magnaporthe grisea* isolates Pg 186, Pg 138, and Pg 45

S. no.	Seed protein of inbred line	Percent reduction in growth (A_{595}) over control		
		Pg 186	Pg 138	Pg 45
1	ICMB 1604	47.5 (43.57)^{n}	9.7 (18.12)^{c}	74.0 (59.37)^{hij}
2	ICMB 100267	29.9 (33.16)^{ef}	13.5 (21.47)^{de}	73.6 (59.11)^{hi}
3	ICMB 101439	26.8 (31.22)^{cd}	36.7 (37.28)^{m}	75.7 (60.47)^{kl}
4	ICMB 101088	35.3 (36.45)^{hij}	12.7 (20.84)^{d}	67.4 (55.2)^{f}
5	ICMB 1608	26.4 (30.92)^{cd}	12.7 (20.84)^{d}	64.9 (53.65)^{d}
6	ICMB 100376	37.5 (37.76)^{jk}	28.8 (32.47)^{k}	76.16 (60.78)^{kl}
7	ICMB 97222	35.9 (36.83)^{hij}	13.6 (21.63)^{de}	76.66 (61.12)^{l}
8	ICMB 1502	27.8 (31.84)^{de}	24.1 (29.42)^{j}	57.8 (49.49)^{c}
9	ICMB 1507	31.7 (34.30)^{fg}	18.0 (25.07)^{gh}	67.5 (55.25)^{f}
10	ICMB 100662	21.8 (27.86)^{b}	6.9 (15.18)^{b}	55.63 (48.24)^{b}
11	ICMR 100014	40.2 (39.36)^{l}	1.5 (6.75)^{a}	68.0 (55.60)^{fg}
12	ICMR 1505	24.8 (29.86)^{c}	15.2 (22.96)^{ef}	74.56 (59.72)^{jk}
13	ICMR 1504	28.4 (32.18)^{de}	21.4 (27.53)^{i}	72.9 (58.65)^{h}
14	ICMR 102088	34.7 (36.11)^{hi}	16.5 (23.94)^{fg}	80.7 (63.97)^{mn}
15	ICMR 100101	39.6 (39.03)^{kl}	28.0 (31.94)^{k}	76.76 (61.19)^{l}
16	ICMR 100016	34.6 (36.03)^{h}	14.6 (22.38)^{de}	63.7 (52.95)^{d}
17	ICMR 100244	36.1 (36.90)^{hij}	13.16 (21.24)^{d}	80.56 (63.85)^{m}
18	ICMR 100004	37.5 (37.76)^{ijk}	23.7 (29.13)^{j}	75.06 (60.05)^{jk}
19	ICMR 100025	61.3 (51.51)^{o}	33.6 (35.44)^{l}	69.1 (56.25)^{g}
20	ICMR 13777	21.5 (27.63)^{b}	33.9 (35.58)^{l}	87.3 (69.13)^{o}
21	ICMR 101012	44.2 (41.65)^{m}	18.5 (25.44)^{gh}	76.4 (60.97)^{l}
22	ICMR 100023	29.6 (32.95)^{ef}	6.46 (14.73)^{b}	67.66 (55.35)^{f}
23	ICMR 100040	33.5 (35.38)^{gh}	34.5 (35.99)^{lm}	81.7 (64.68)^{n}
24	ICMR 100098	27.8 (31.82)^{de}	20.0 (26.58)^{hi}	66.1 (54.42)^{e}
25	ICMR 100021	19.2 (25.96)^{a}	5.66 (13.74)^{b}	53.8 (47.2)^{a}
	Mean	33.4	18.5	71.4
	LSD ($P=0.05$)	1.472	1.459	0.706

Values in parentheses are arcsine transformations

Values followed by the same small letter within a column are not significantly different ($P=0.05$) (Duncan's multiple range test)

activity levels of chitinases are in a positive association with blast disease (ρ 0.5) rather than downy mildew (ρ 0.03). Conversely, the correlation constants of glucanase and cellulase activities are relatively positive towards downy mildew resistance (glucanases ρ 0.28; cellulases ρ 0.44) over blast disease resistance (glucanases ρ 0.12; cellulases ρ − 0.03) Similarly, ICMB 1604, ICMB 100267, ICMB 101439, ICMB 101088, ICMR 1504, ICMR 102088, ICMR 100101, and ICMR 100040 lines with prominent levels of cystatins (36.2–58.5 units/mg), chitinases (9.7–16.3 units/ml), and β-1,3-glucanases (21.8–32.9 units/mg) exhibited a relatively high degree of resistance against more than one pathotype-isolates of the foliar blast disease in greenhouse screenings. Conversely, lines ICMB 1502, ICMB 1507, ICMB 100662, ICMB 1608, and ICMR 100098 with low levels of cystatins (4.3–11.6 units/mg), chitinases (6.6–8.5 units/ml), and β-1,3-glucanases (7.3–18.2 units/mg) exhibited moderate to susceptible reaction against blast disease. Such apparent positive consistency was also observed in lines ICMB 101439, ICMR 100014, ICMR 1505, ICMR 102088, ICMR 100101, ICMR 100016, and ICMR 100244 with activity levels of β-1,3-glucanases (20.1–32.9 units/mg), cellulases (1.1–3 units/mg), and/or cystatins (13–58.5 units/mg) and downy mildew resistance. While downy mildew susceptible lines ICMB 100662 and ICMR 100021 exhibited a relatively low levels of cystatins (6.8, 8.2 units/mg) as well as PR hydrolases (β-1,3-glucanases, 7.8–14.9 units/mg; cellulases, 0.21–0.29 units/mg). On the contrary, ICMB 101088 exhibited moderate to susceptible reaction against *S. graminicola* isolates, though it exhibited prominent cellulase activity (4.1 units/mg) indicating differential response of these biochemicals in the host genotypes towards imparting stress tolerance.

On the whole, resistance exhibited by lines ICMB 101439, ICMR 102088, ICMR 100101, and ICMR 100040 towards blast and downy mildew diseases is in accordance

Table 5 Efficacy of seed proteins of biofortified pearl millet inbred lines in reducing downy mildew incidence (%) in susceptible lines ICMP 451 against *Sclerospora graminicola* isolates Sg 576 and Sg 409, and in 7042R against isolate Sg 445 following seed treatment

S. no.	Seed protein of inbred line	Downy mildew incidence (%)		
		Sg 576	Sg 409	Sg 445
1	ICMB 1604	56.1 (48.49)^{jk}	60.26 (50.93)^{i}	20.0 (26.57)^{ef}
2	ICMB 100267	54.4 (47.5)^{hi}	52.16 (46.24)^{h}	12.3 (20.56)^{b}
3	ICMB 101439	48.2 (43.99)^{f}	44.5 (41.86)^{c}	21.5 (27.63)^{fg}
4	ICMB 101088	44.9 (42.08)^{e}	46.26 (42.86)^{cd}	17.1 (24.36)^{d}
5	ICMB 1608	54.3 (47.45)^{hi}	50.5 (45.28)^{gh}	8.3 (16.73)^{a}
6	ICMB 100376	40.6 (39.6)^{c}	45.74 (42.56)^{cd}	16.5 (23.96)^{cd}
7	ICMB 97222	44.1 (41.61)^{de}	41.0 (39.81)^{b}	17.3 (24.58)^{d}
8	ICMB 1502	57.2 (49.16)^{kl}	47.4 (43.52)^{de}	33.0 (35.05)^{k}
9	ICMB 1507	59.2 (50.32)^{m}	39.4 (38.85)^{ab}	31.3 (34.02)^{jk}
10	ICMB 100662	53.1 (46.8)^{h}	49.3 (44.6)^{fg}	36.09 (36.92)^{l}
11	ICMR 100014	43.2 (41.09)^{d}	48.2 (43.96)^{ef}	18.34 (25.35)^{de}
12	ICMR 1505	54.8 (47.74)^{hij}	46.26 (42.86)^{cd}	24.0 (29.31)^{hi}
13	ICMR 1504	55.4 (48.08)^{ij}	59.2 (50.3)^{i}	13.25 (21.31)^{b}
14	ICMR 102088	39.6 (39.00)^{bc}	39.6 (39.02)^{ab}	15.0 (22.78)^{c}
15	ICMR 100101	43.9 (41.51)^{de}	48.5 (44.14)^{ef}	15.1 (22.87)^{c}
16	ICMR 100016	53.2 (46.84)^{h}	39.0 (38.65)^{a}	16.5 (23.99)^{cd}
17	ICMR 100244	48.1 (43.91)^{f}	51.4 (45.78)^{h}	21.2 (27.4)^{fg}
18	ICMR 100004	50.6 (45.32)^{g}	49.2 (44.55)^{efg}	8.5 (16.91)^{a}
19	ICMR 100025	35.5 (36.59)^{a}	49.5 (44.73)^{fg}	29.7 (33.01)^{j}
20	ICMR 13777	54.4 (47.5)^{hi}	51.8 (46.01)^{h}	22.2 (28.07)^{gh}
21	ICMR 101012	58.2 (49.74)^{lm}	38.8 (38.51)^{a}	24.38 (29.58)^{i}
22	ICMR 100023	54.5 (47.59)^{hij}	46.25 (42.85)^{cd}	29.5 (32.89)^{j}
23	ICMR 100040	51.2 (45.69)^{g}	48.1 (43.93)^{ef}	31.6 (34.19)^{jk}
24	ICMR 100098	38.4 (38.29)^{b}	45.2 (42.25)^{c}	24.0 (29.35)^{hi}
25	ICMR 100021	62.5 (52.24)^{n}	49.3 (44.58)^{fg}	30.7 (33.66)^{j}
26	Control	91.9 (73.59)^{o}	60.6 (51.13)^{i}	42.2 (40.49)^{m}
	Mean	51.8	48.0	22.3
	LSD ($P=0.05$)	0.8461	0.9262	1.27

Values in parentheses are arcsine transformations

Values followed by the same small letter within a column are not significantly different ($P=0.05$) (Duncan's multiple range test)

with the relatively elevated levels of cystatins (45.5–58.5 units/mg), β-1,3-glucanases (24.5–32.9 units/mg), and/or chitinases (9.7–16.3 units/ml) and/or cellulases (0.7–3 units/mg) in these lines. Conversely, lines, ICMB 100662 and ICMR 100098 with low level of cystatins (4.3–8.21 units/mg), β-1,3-glucanases (7.8–9.0 units/ml), and/or chitinases (6.6–8.5 units/ml), and/or cellulases (0.19–0.29 units/mg) exhibited relatively susceptible reaction against both the diseases. The synergistic contribution of PR proteins/hydrolases towards pathogen resistance is well reported. Transgenic tobacco expressing chitinase gene alone has not shown any enhanced resistance against yellow leaf blotch pathogen, though the growth of fungus was very sensitive under in vitro conditions (Neuhaus et al. 1991). However, simultaneous expression of chitinases (class I) and β-1,3-glucanase genes in transgenic tomato showed enhanced resistance (36–58% reduction in disease severity) against Fusarium wilt of tomato suggesting their cumulative role in disease resistance, while such increase in resistance was not observed with the expression of either of the gene (Dolatabadi et al. 2014). Similarly, the defensive role of PIs in blast resistance is supported by the strong induction of these proteins in blast-resistant rice during infection (Han et al. 2004). Nevertheless, the total seed protein contents of seeds are neither in accordance with disease resistance nor in activity levels of defense proteins (Tables 1, 2, 3).

Furthermore, seed proteins were evaluated for antifungal activity against the radial growth of *M. grisea* pathotype-isolates Pg 45, Pg 138, and Pg 186 on OMA media. The observed variability in growth retardation of Pg 138 (9–57%) compared to other isolates Pg 45 (10%) and Pg 186 (13%) could be due to differential expression of secreted proteases as well as in proportional existence of polysaccharides such as chitin, glucan, and also other cell wall components such

as lipids and proteins among isolates (Machin et al. 2008; Fig. 2 and Supplementary Fig. 1). The effect of seed proteins on spore germination and initial fungal growth (48 h) was further evaluated by a quantitative spectrophotometric assay (Broekaert et al. 1990). The spore germination stage is very sensitive and prone to inhibition than fungal mycelium. Compounds with less or no inhibitory activity against mycelial growth may also act as potent inhibitors against spore germination as evidenced in the strobilurin class of fungicides (Balba 2007; Sauter et al. 1995). They exhibit target-specific inhibition against spore germination, thereby preventing early penetration of the host with no or little effect on later stages of fungal growth. In contrast, some inhibitors may exhibit strong inhibition against mycelial growth with poor or no inhibitory effect on spore germination (Stehmann and Waard 1996). In this context, the present study resulted in moderate-to-high retardation in spore germination and initial fungal growth of Pg 45 (53.8–87.3%) followed by Pg 186 (19.2–61.3%) and Pg 138 (1.5–36.7%), as represented in Table 4. Similar kind of inhibition in fungal spore germination has been reported for PIs as well as PR hydrolases (Ji and Kuc 1996; Dunaevsky et al. 1997).

The efficacy of seed proteins was tested for the reduction in downy mildew disease incidence (against Sg 409, Sg 445, and Sg 576) by treating seeds of pearl millet lines ICMP451 and 7042R (with differential susceptibility to pathogen isolates) with the seed proteins of high Fe and Zn lines used in the present study (Table 5). Low to a significant reduction in disease incidence was observed against Sg 409 (0.6–36%), Sg 576 (32–61%), and Sg 445 (14–80%) compared to untreated seeds. PIs as well as PR hydrolases have been found to act as positive defense regulators against oomycete pathogens (Kini et al. 2000; Balasubramanian et al. 2012). Rapid induction of PI gene was identified upon oomycete pathogen *Phytophthora parasitica* interaction with tobacco, and silencing this gene compromised resistance to the pathogen (Silva et al. 2013). Also, the accumulation of PIs in potato tubers upon infection with zoospores of *P. infestans* was observed and found to suppress the germination of zoospores as well as the growth of *P. infestans* hyphae (Valueva et al. 2003). The observed results are well corroborated with the *Lactuca sativa*-mediated enhanced resistance of pearl millet against downy mildew disease (Mythrashree et al. 2013). The observed reduction in disease incidence and elevated defense response in the present study might be due to the priming effect of partially degraded oligosaccharide elicitors of the pathogen cell wall by the action of β-1,3-glucanases and cellulases along with the direct hydrolysis (Kombrink et al. 2001).

Conclusion

Seed proteins from the selected pearl millet lines, rich in Fe and Zn content, exhibited significant variation in the level of protease inhibitors against cysteine proteases, and PR hydrolases such as chitinases, β-1,3-glucanases, and cellulases which are possibly playing a synergistic role in defense against blast and downy mildew diseases. The variable levels of these enzymes in pearl millet seeds can be used as biochemical markers to screen pearl millet lines for their differential resistance levels against blast and downy mildew diseases. The biochemical basis of resistance elucidated in the present study will be helpful in the exploitation of novel defense strategies in the pearl millet disease resistance breeding program.

Author contribution statement MS: designed and executed experiments, and wrote the manuscript; NN: helped in performing greenhouse screenings, TS: helped in the execution of chitinase activity; MG: developed seed material, RS: provided lab facilities; MG and RS: provided critical suggestions in designing experiments, supervised the experimental work, and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials (data transparency) Authors agree to keep the data available.

Compliance with ethical standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Consent to participate (include appropriate statements) Authors agree to participate in the review forum.

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