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Conserved nature of *Helicoverpa armigera* gut bacterial flora on different host plants and in vitro interactions with PI proteins advocates role in host digestive physiology

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ABSTRACT

Helicoverpa armigera is anxious insect pest of agricultural crops. Array of defensive molecules in host plants and extensive use of chemical insecticides are unable to cease the attack incidences. Gut bacterial communities are found to contribute in various physiological activities in most of the arthropods. In the current study the bacterial communities were isolated from gut of *H. armigera* feeding on three host plants (Pigeonpea, Chickpea and Cotton) by culture dependent and culture independent methods. Predominant bacterial communities were identified by terminal restriction fragment length polymorphism (TRFLP). Three dominant phylotypes namely proteobacteria, actinobacteria and firmicutes were identified by TRFLP and found to conserve on different host plant selected. Five *Bacillus* species namely *Bacillus* sp. JR14, *Bacillus* sp. YP1, *Bacillus safensis* CG1, *Bacillus subtillis* KAVK2 and *Bacillus megaterium* 47N were purified by culture dependent method and identified by 16S rRNA sequencing. Among all identified *Bacillus*, *Bacillus* sp. YP1 strain was found to be potent protease producer as assisted by dot-blot assay and in vitro solution assays. The in vitro interactions of these proteases with host plant PIs were studied by reverse zymography and gel X-ray contact print (GXCP) analysis. Reduction in activity of PIs and degradation pattern of PI bands on gels in presence of trypsin and protease extract of *Bacillus* sp. YP1 indicates inactivation of PIs. Thus, conserved nature and in vitro response to PI proteins advocates role of gut bacterial flora in *H. armigera* digestive physiology.

1. Introduction

The symbiotic associations of gut bacteria with the host are imperative for the host fitness (Clemente et al., 2012). Gut bacterial contribution in metabolic activities through multitrophic interactions are essential for the survival of insect (Douglas, 1992; Dillon and Dillon, 2004; Pilon et al., 2013). The gut bacterial communities are found to contribute in nutrition, reproduction and pheromone biosynthesis (Lundgren and Lehman, 2010; Tartar et al., 2009; Dillon and Dillon, 2004). These bacterial communities are efficient and found to metabolize toxicants present in the ingested food (Douglas, 2009; Engel and Moran, 2013). Experimental studies have been showing reduced fecundity and growth of the insect in the absence of gut bacterial communities (Visôtto et al., 2009a). Mutual interaction between the gut bacterial communities and insect is developing more interest among researchers and could be study for its application in pest control.

H. armigera is the polyphagous insect pest and leading biotic constrain for food legumes (Sharma et al., 2008). The host range for this insect pests raked around 300 crops worldwide (Arora et al., 2005). A complex and diverse proteolytic environment in the gut is responsible for polyphagy of this insect (Gatehouse et al., 1997; Chougule et al., 2005; Srinivasan et al., 2006; Angelucci et al., 2008; Chikate et al., 2013). This proteolytic arsenal helps *H. armigera* to acclimatize proteinaceous defense employed

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by the host plants (Bown et al., 2004; Chikate et al., 2013). These proteolytic activities may suppress by antibiotics as observed in *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) (Visôtto et al., 2009a). The above finding suggests possible involvement of gut bacterial proteases in the adaptation of insect to host plant protease inhibitors (PIs). Furthermore gut bacterial communities resistance to organophosphate pesticides were also reported (Natarajan, 2012). Further exploration of insect gut bacterial communities in context with its ability to produce enzyme arsenal and assistance to host insect against plant defense molecules (PIs) is needed to design effective strategies against *H. armigera*. With this goal in mind here we are reporting the presence of conserved bacterial communities on different host plants (pigeonpea, chickpea and cotton). Among those five bacterial strains were identified as protease producer. In vitro interaction of host PIs (pigeonpea, chickpea, mungbean and soybean) was studied with strain having large proteases producing potential. Cultivation and identification of bacterial communities were employed by both culture dependent and independent methods. Proteolytic and PIs activity was explored qualitatively and quantitatively by using techniques such as dot blot assay, solution assay and zymography analysis, reverse zymography analysis and gel X-ray contact print technique (GXCP).

2. Materials and methods

2.1. Collection of larvae

The 2nd instar larvae of *H. armigera* were separately collected from cotton, pigeonpea and chickpea fields located near Aurangabad city, Maharashtra, India. For microbial analysis after shifting, one group (10 larvae in each group) of 2nd instar larvae was moved from one host to other in following manner. Two groups of larvae native to pigeonpea were transferred to cotton and chickpea and two groups of larvae native to cotton transferred to pigeonpea and chickpea. Finally two groups from chickpea are shifted to cotton and pigeonpea. These larvae with altered host (grown naturally) allowed reared up to the 6th instar in departmental field.

2.2. Culture independent identification of gut bacterial communities

For identification of gut bacterial communities, the guts of larvae feeding on its native host plants and altered host plants were assessed for having microbial communities. The 10 individual larvae of 6th instar were dissected with alcohol-sterilized dissecting tools and guts were removed carefully. The isolated guts were placed in sterile micro centrifuge tubes on the ice. DNA was extracted with a QIAamp DNA Stool Mini Kit (QIAGEN, USA). The extracted DNA was quantified with Eppendrof BioSpectrometer® and stored at −20 °C.

The extracted DNA was subjected to 16S rDNA fragment amplification by PCR using the 27F 5′-AGAGTTTGATCMTGGCTCAG-3′ and 1492R 5′-TACGGYTACCTTGTTACGACT-3′primers (Sigma) (Weisburg et al., 1991). The forward primer 27F is labeled with 6-carboxyfluorescein (6-FAM). The 50 μl PCR mixture consisted of 1 μl (100 ng) of template DNA, 4 μl (3 μM) of the primers, 5 μl (2.5 mM) dNTP (Sigma), 0.2 μl (5 U) Taq DNA polymerase (Sigma) and 5 μl 10X PCR buffer and 29.8 μl MiliQ water. The PCR conditions were initial melt at 95 °C for 3 min (1 cycle); 94 °C for 30 s, 53 °C for 30 s and 72 °C for 60 s (up to 35 cycle); with a final extension at 72 °C for 10 min and infinite hold at 4 °C. The resulting amplicons were purified by Sigma PCR cleanup kit.

The purified PCR products were digested with restriction enzymes, *AfaI (RsaI)* and *MspI* separately procured from Invitrogen. The digestion mixture contained 30 μl PCR products, 1 μl enzyme, 5 μl buffer and the total volume was made up to 50 μl with distilled water. The mixture was incubated at 37 °C for overnight in a PCR machine.

About 5 μl of digested mixture was taken in a PCR tube, followed by addition of 125 μl of 100% chilled ethanol, 5 μl of Sodium acetate (3 M, pH 5.2) and 5 μl (0.5 M) EDTA. The mixture was incubated at room temperature for 15 min and pelleted down at 12,000 rpm at 4 °C for 15 min by using centrifuge. The pellet was washed with 35 μl 70% ethanol. The desalted pellet was dried and mixed with 10 μl of Hi-di formamide and 0.25 μl of internal size standard (1200Liz – Genescan, Applied Biosystems, USA), denatured for 5 min at 95 °C and maintained on ice until its application into an auto sampler for sequencing.

The samples were analyzed on a 3500 Genetic Analyzer (Applied Biosystems, USA). The data obtained was processed with Gene Mapper software v 5.0. The abundance measurement was calculated using the area under the curve of each fragment peak. The data obtained from Gene Mapper was analyzed through Microbial community analysis III (MiCA) (http://mica.ibest.uidaho.edu/-pat.php). The possible microbial profiles corresponding to fragment length and abundance were obtained through MiCA. The similarity in the bacterial composition was determined using the alignments of T-RFLP profiles followed by pair wise comparison analysis.

2.3. Culture dependent purification and characterizations of gut bacteria

As initial effort to understand importance of bacterial communities in *H. armigera* digestive physiology (in the protease production), we used culture dependent approach. The gut of the 6th instar *H. armigera*, feeding on pigeonpea plant homogenized in 1 ml 1X PBS (Phosphate buffered saline, pH 7.0) followed by sonication (at 30 Amplitude, 1 s pulse). The homogenate was serially diluted in nutrient broth (NB) from 10^{-1} to 10^{-9}. About 100 μl of dilution (10^{-9}) was placed on NB plates and incubated for 72 h at 37 °C. The bacterial colonies appeared were observed visually and five different singlet colonies were picked and grown on different NB plates to obtain pure bacterial cultures.

The DNA was extracted from pure bacterial cultures separately and amplified by PCR. The resulting amplicons were purified by Sigma PCR cleanup kit and sequenced using ABI 3500 genetic analyzer and big dye termination 3.2 v kit. The resulting sequence of 16S rRNA were analyzed with Sequencher 5.1 software and compared to gene libraries using blastn tool. The corresponding similar sequences were downloaded and phylogenetic tree was constructed using a Maximum likelihood method in MEGA 5.2 with 1000 bootstrap replicates using Kimura 2 parameter (k2 model) nucleotide substitution model.

The pure bacterial cultures isolated from gut of the 6th instar *H. armigera*, feeding on pigeonpea plant were subjected to protease production on nutrient broth supplemented with 1% skimmed milk powder separately. Protease production was carried out at pH 9.0 for 48 h at 37 °C. Each broth was centrifuged at 5000 rpm and the supernatant was used as a crude source of protease.

2.4. Qualitative and quantitative analysis

The gelatinolytic activity of the each gut bacterial supernatant of proteases was evaluated using gelatin coated X-ray film (Pichare and Kachole, 1994). About 20 μl supernatant was spotted on X-ray film separately and incubated at 37 °C for 10 min. The clear zone of gelatin hydrolysis was observed visually.

For zymographic analysis, bacterial supernatant were separated electrophoretically on 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) incorporated with 0.1% gelatin (from porcine skin; Sigma) as a substrate.

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About 75 μg of protein from each bacterial supernatant was loaded in lane of gel separately. After the run, sodium dodecyl sulphate (SDS) was removed by washing the gel twice with 2.5% Triton-X 100 for 40 min. The gel was washed with distilled water thrice and incubated overnight in 20 mM Tris-HCl (pH 9.0) at 37 °C. The gel was then stained with coomassie blue R-250 and the zones of proteolysis were identified.

Total protease activity was determined using the azo-casein (Sigma) as substrate. Mixture containing 250 μl of 0.5% azo-casein (prepared in 0.1 M Tris-HCl pH 7.8), 250 μl buffer and 100 μl of bacterial supernatant was incubated for 30 min at 37 °C. About 300 μl Trichloroacetic acid (TCA, 5% w/v) was added to the mixture to stop the reaction. The mixture was centrifuged at 10,000 rpm for 10 min at 4 °C. Equal volume of 1 N NaOH was added to the supernatant and the activity was measured at 440 nm. The blank used for this assay was prepared by incubating a mixture of the bacterial supernatant and buffer for 30 min at 37 °C followed by addition of TCA and azo-casein.

2.5. Crude seed PIs preparation and interactions study

It is believed that insect gut bacterial communities are involved in adaption of host insect on plants enriched in plant defense components like PIs. This adaptation is attributed to secretion of PIs resistant proteases or degradation of PIs molecules in the alkaline environment of gut of insects. To analyze in vitro interaction of isolated gut bacterial proteases and crude PIs from host plants, PIs from seeds of pigeonpea, chickpea, mungbean and soybean extracted. The seeds were crushed and fine powders were obtained by using mortar and pestle/mixer grinder. The powder was defatted and depigmented with hexane and acetone respectively followed by air drying. For protein extraction, 5 g of each seed powder was stirred in 10 volumes of 0.85% saline for 2 h, followed by centrifugation at 5000 rpm. For partial purification obtained supernatant was precipitated with three volumes of chilled acetone. Precipitate obtained was dried and used as a source of PIs for further studies. The quantative estimation of protein was

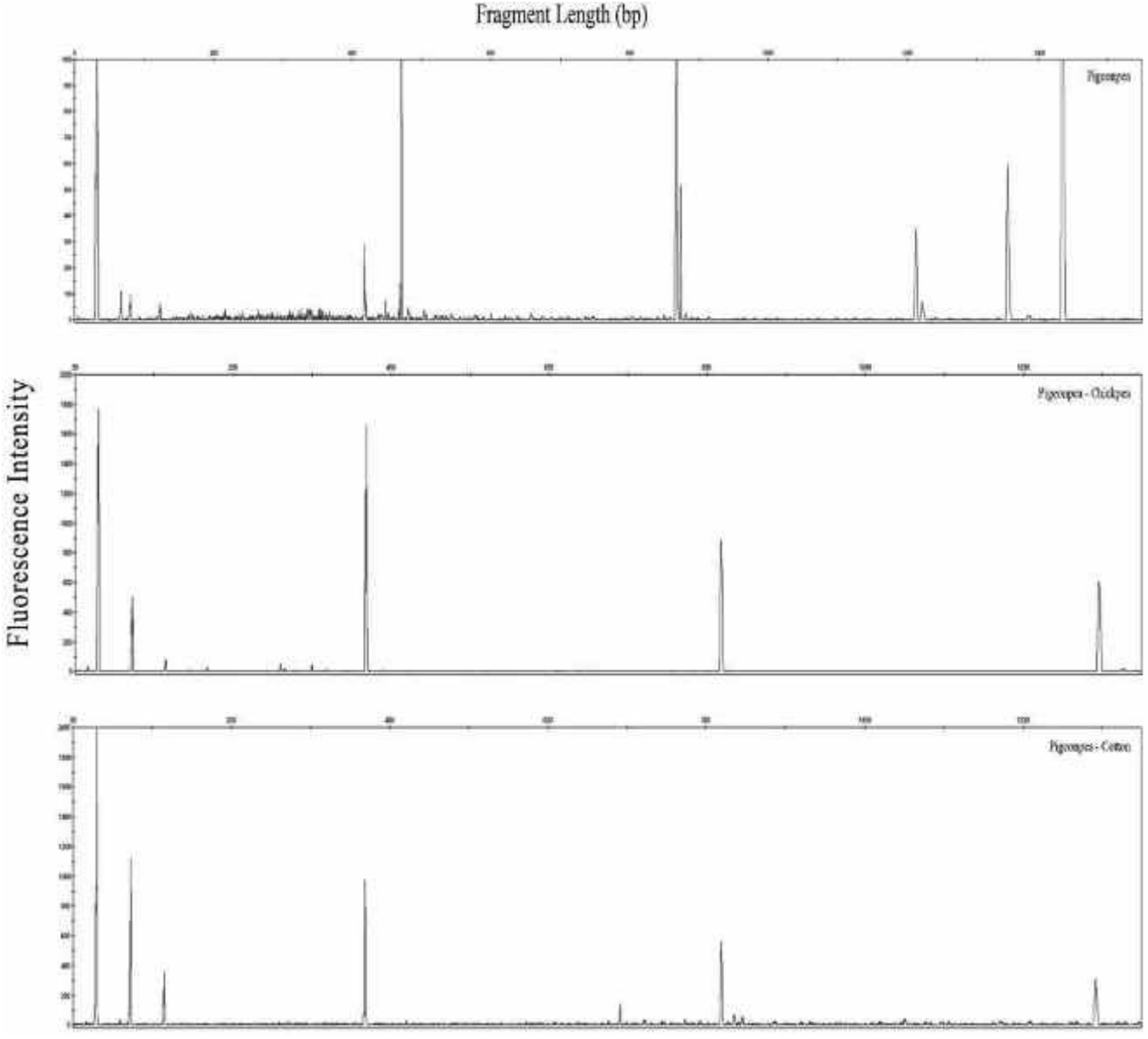

Fig. 1. T-RFLP profiles of *H. armigera* gut bacterial communities: on pigeonpea, upon shift on pigeonpea to chickpea and pigeonpea to cotton.

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determined by the method of Lowry et al. (1951) using Bovine serum albumin as the standard.

To analyze interactions between bacterial proteases and host seed PIs, host crude seed proteins digested by trypsin and gut bacterial proteases. About 100 μg of seed protein from each host (Pigeonpea, chickpea, mungbean and soybean) was separately subjected to digestion by equivalent (0.1 mg) concentration of trypsin, *Bacillus* sp. YP1 bacterial proteases (with highest activity) and mixture containing equal protein concentration of trypsin and *Bacillus* sp. YP1 bacterial proteases. The digestion was carried out for 4 h at 37 °C. The resulting samples were resolved on 10% SDS-PAGE for protein profiling. To analyze remaining PIs activity from host seed protein against respective proteases, digested samples were further preceded for GXCP (Pichare and Kachole, 1994) and reverse zymography analysis (Le and Katunuma, 2004).

2.6. Statistical analysis

The statistical analysis of all the data obtained by T-RFLP experiments was performed using PAST (Paleontological Statistic Software ver. 1.88). All solution assays were conducted and analyzed in triplicate. Means and standard deviations were calculated and compared. Analysis was performed using Microsoft Excel.

3. Results and discussions

Insects are most abundant and diverse eukaryotes on the earth (Dillon et al., 2000; Grimaldi and Engel, 2005). They are largely reliant on the gut bacterial communities for basic functions (Engel and Moran, 2013). Gut bacterial communities are responsible for modulating behavior of the host insect (Lewis and Lizé, 2015). Thus, the understanding nature and its biological role in host insect physiology are imperative to control of insect population in agriculturally important crops.

H. armigera is insect pest, rapidly overcoming present control measures counting chemical, microbial insecticides and genetically improved plants (Kranthi et al., 2002; Rajagopal et al., 2009). Earlier gut bacterial communities in the context with its ability to secrete enzymes in insect gut were reported from several insect

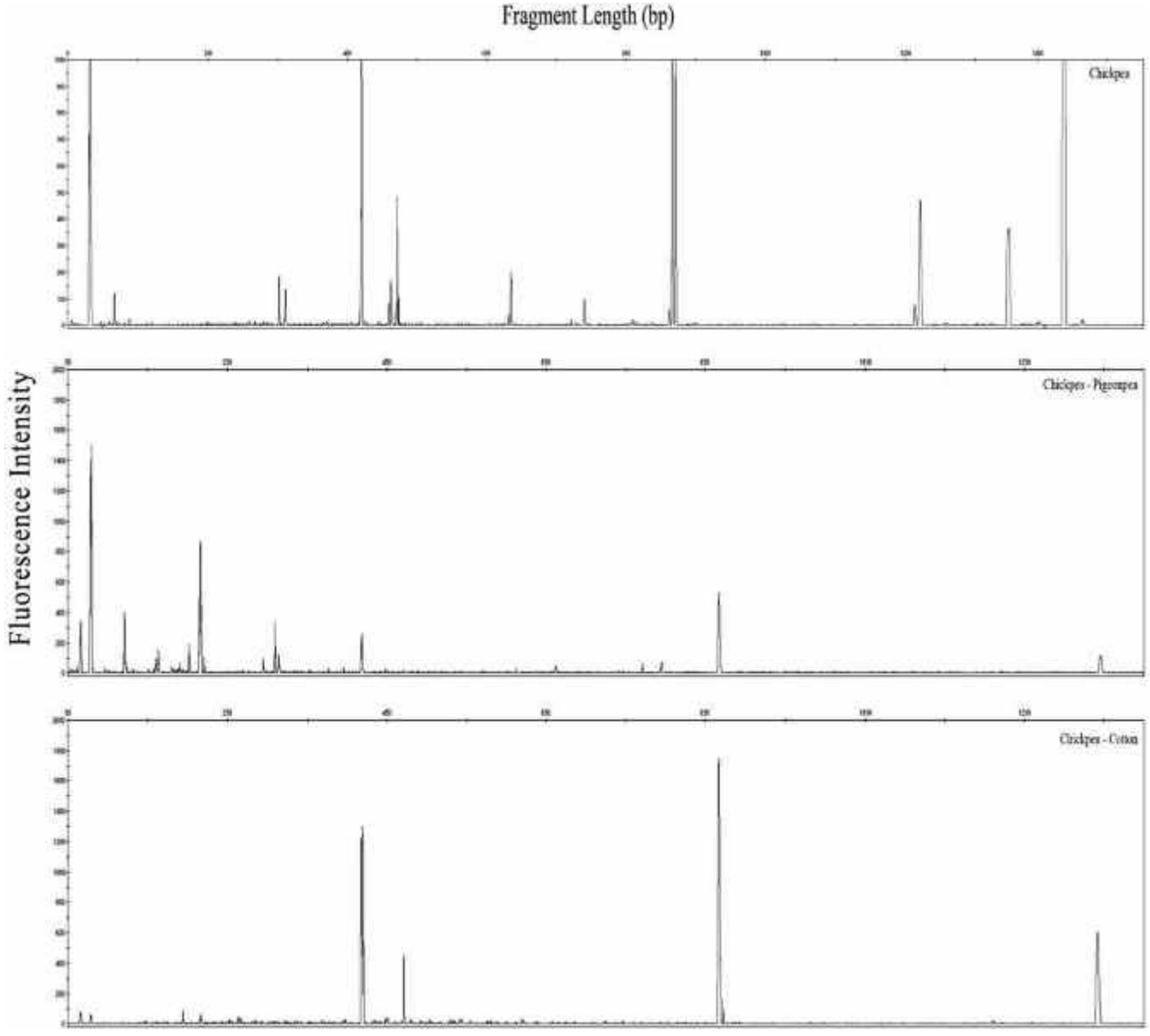

Fig. 2. T-RFLP profiles of *H. armigera* gut bacterial communities: on chickpea, upon shift on chickpea to pigeonpea and chickpea to cotton.

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species including *H. armigera*. As per author literatures scrutiny following points could not explored in detail, (a) The response of these bacterial communities to the different host plants of insects when shifted from one host to other (b) Detailed understanding of biochemical basis of interactions of these gut bacterial secreted proteases with host plant defense molecules such as PIs. These studies will provide insight to design competent alternative to existing control measures against *H. armigera*. With this goal in mind here we (a) isolated the gut bacterial communities of *H. armigera* by culture independent and culture dependent methods (b) The variation in gut bacterial communities associated with shifting were analyzed (c) Bacterial colonies isolated by the culture dependent method were analyzed for protease production and (d) The in vitro interactions of these bacterial proteases with host plant PIs studied.

3.1. Culture independent analysis

The 16S rDNA gene based culture-independent molecular ecological investigation generates improved and inclusive picture of bacterial communities living inside the gut of insects (Yun et al., 2014). The 16S rDNA of bacteria isolated from the gut of insect reared on host plants (Pigeonpea, Chickpea and Cotton) and reared on other host plants after shift were successfully extracted, amplified, purified and detected by 1% agarose gel. The analysis of variation in bacterial populations is frequently done by gene sequence polymorphisms using restriction enzymes (Liu et al., 1997). T-RFLP analysis was used to study the changes in bacterial community upon shifting. Two restriction enzymes, *AfaI (RsaI)* and *MspI* were used for restriction digestion separately. Although it is reported that the usage of more than one restriction enzyme enables the resolution of bacterial populations, but not in term of phylotypes richness and diversity estimates based on diverse indices (Liu et al., 1997). Sample data comprise of the size in base pair and peak area for each TRF peak in electrophoregrams. One TRF is considered as a phylotypes while each peak area displays the relative abundance of the TRF. The average fragments size was obtained in the range of 54.52–1348.79 bp in the gut of larvae feeding on different host plant and upon shifting (Figs. 1–3). TRFLP analysis showed the presence of significant diversity in gut bacterial population of the larvae collected for each host but slight variation were induced after shift different host. Proteobacteria, Actinobacteria and Firmicutes bacterial communities were found to be predominant in the gut of all selected larvae collected. Besides, some uncultured bacterial species were observed on in all tested gut samples (Table 1).

The order of bacterial species richness and diversity is given in Table 2. The richness and diversity was found to be in the increasing order of pigeonpea, chickpea and cotton. Upon shifting of *H.*

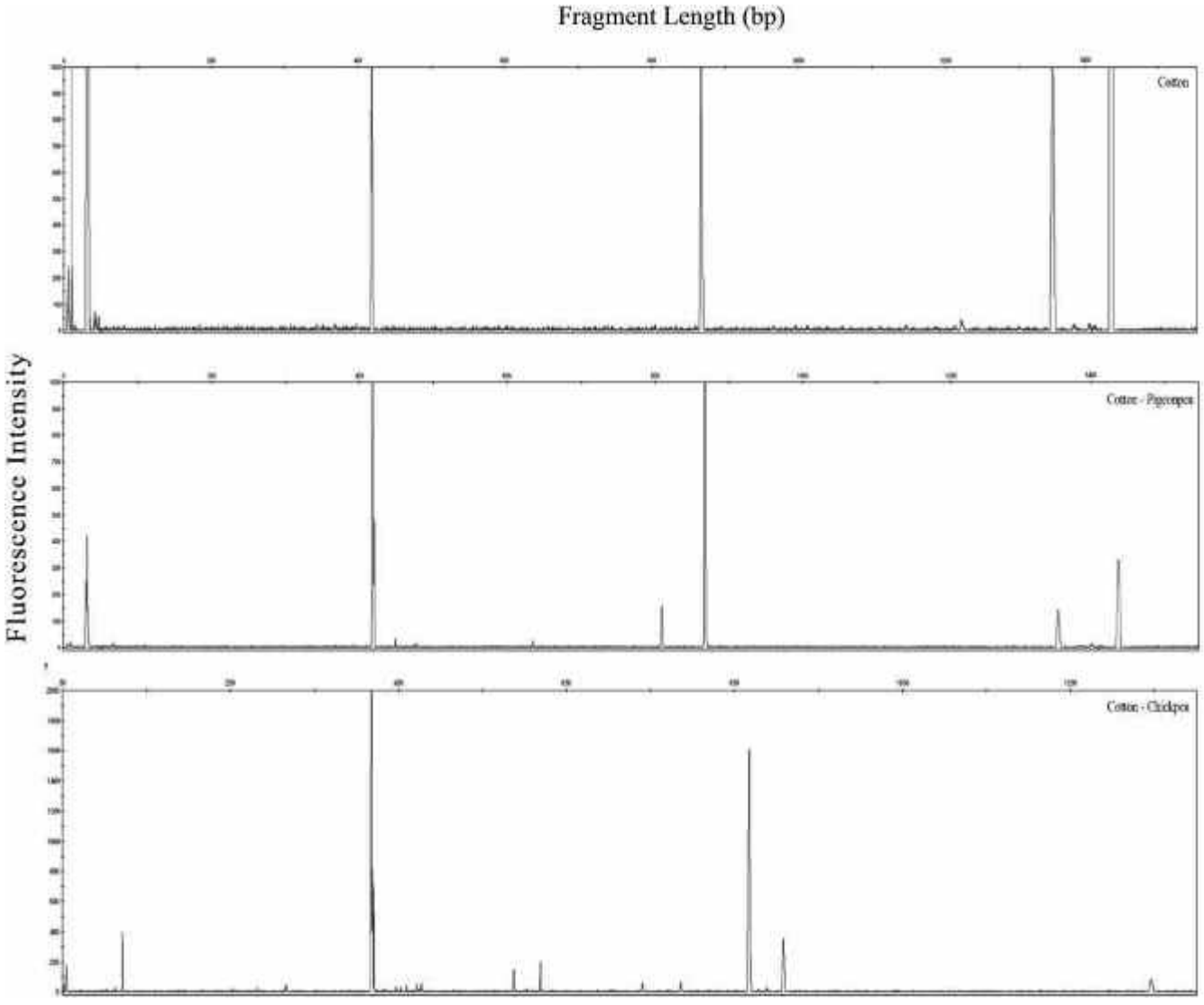

Fig. 3. T-RFLP profiles of *H. armigera* gut bacterial communities: on cotton, upon shift on cotton to pigeonpea and cotton to chickpea.

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Table 1
PAT analysis for 16S rDNA phylotypes found in gut of *H. armigera*.

TRF size (bp)		Phyla
Afa-I	Msp-I	
66.56	66.21	Proteobacteria
78.69	72.09	Uncultured bacterium
79.83	572.45	Uncultured bacterium
121.02	81.09	Uncultured bacterium
122.49	194.44	Uncultured bacterium
309.39	487	Proteobacteria
419.06	437.68	Proteobacteria
420.64	437.68	Proteobacteria
459.82	66.21	Actinobacteria
462.26	66.21	Actinobacteria
470.05	487.35	Proteobacteria
473.58	444.16	Proteobacteria
907.85	572.45	Firmicutes

Table 2
Bacterial community richness and diversity analysis of *H. armigera* gut sample from different hosts (Diversity indices were calculated on the basis of T-RFs data taking the % relative intensities of different peaks into consideration). *S* – Phylotypes richness, *H′* – Shannon-Weiner index, 1-*D* – Simpson's index of diversity.

Host	*S*	*H′*	1-*D*	Evenness
Pigeonpea	58	3.88	0.975	0.836
Pigeonpea-Chickpea	16	2.43	0.883	0.71
Pigeonpea-Cotton	4	1.26	0.686	0.884
Chickpea	22	2.95	0.942	0.874
Chickpea-Pigeonpea	16	2.49	0.894	0.756
Chickpea-Cotton	9	1.97	0.84	0.802
Cotton	4	1.26	0.686	0.884
Cotton-Pigeonpea	9	2.01	0.853	0.831
Cotton-Chickpea	6	1.68	0.795	0.898

armigera from pigeonpea to chickpea, species richness and diversity declined from 58, 3.88 to 16, 2.43. The decreased in species richness and diversity also observed upon shifting of *H. armigera* from chickpea to cotton. Further analysis of T-RFLP data using the Correspondence Analysis (CA) revealed total 36.50% of variance between the samples. These results are in agreement with the results reported earlier with *H. armigera* gut bacterial communities (Xiang et al., 2006; Gayatri et al., 2012).

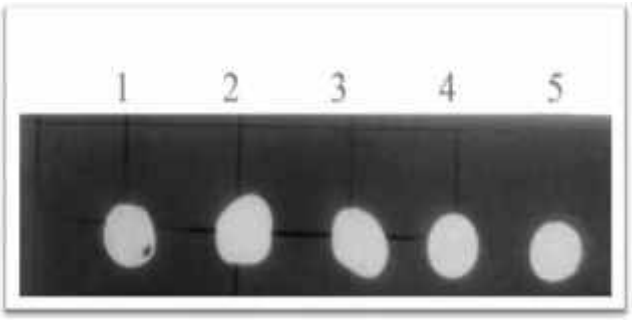

Fig. 5. Dot-blot assay - Proteolytic (Gelatinolytic) activity of serine proteases of *Bacillus* species. (1) *Bacillus* sp. JR14, (2) *Bacillus* sp. YP1, (3) *Bacillus safensis* strain CG1, (4) *Bacillus subtillis* strain KAVK2, (5) *Bacillus megaterium* strain 47N.

The slight variation in bacterial communities to induced shift suggests that the conserved common bacterial communities may contribute to host survival in stress conditions. Earlier studies also demonstrated presence of common bacterial flora in the insect gut irrespective of season, crop, environment and geographic location (Gayatri et al., 2012).

3.2. Culture dependent analysis

Comprehensive molecular analysis of the gut bacterial communities with respect to contribution to host in its growth and development is prerequisite to design competent control measures to resist insect infestation in field crops (Kazzazi et al., 2005). Five bacterial communities were isolated and purified from the insect gut of larvae from host plants i.e. pigeonpea, chickpea and cotton by culture dependent method. Bacterial communities were identified using 16S rRNA sequencing as *Bacillus* sp. JR14, *Bacillus* sp. YP1,

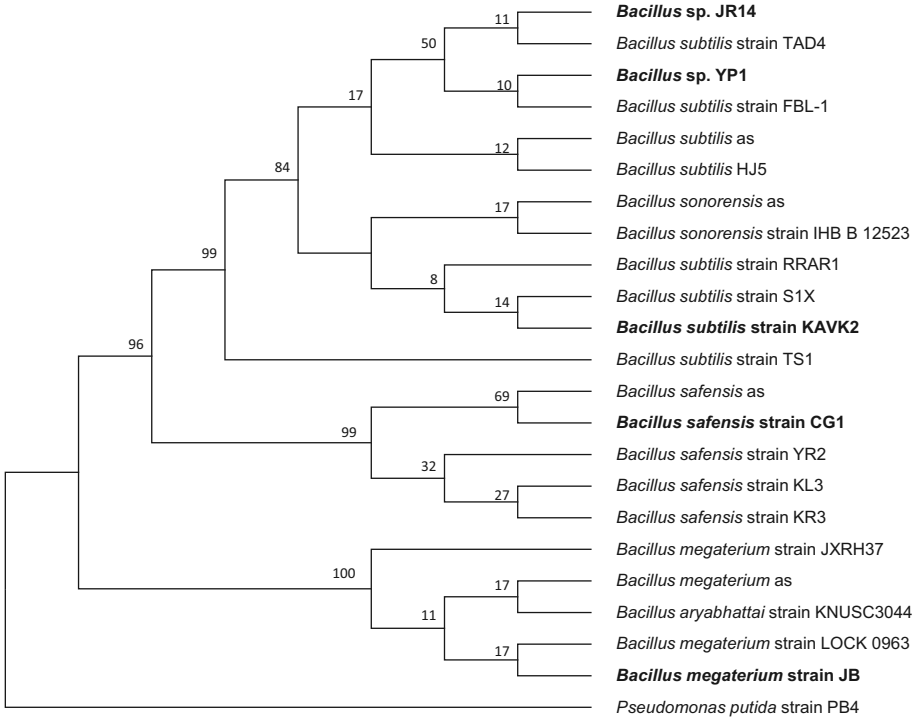

Fig. 4. Phylogenetic tree for partial 16r RNA gene of *H. armigera* gut isolates.

Bacillus safensis CG1, *Bacillus subtillis* KAVK2 and *Bacillus megaterium JB*. The phylogenetic analysis constructed using MEGA 5.2 showed their relatedness (Fig. 4).

Natural defense strategies of host plants include expression of defense proteins like PIs. These proteins have tendency to block proteases expressed within gut environment. The composite protease system expressed in gut of *H. armigera* is responsible for weak response of protease inhibitors based strategies (Patankar et al., 2001). The gut bacterial communities may part of this adaptation or it may releases proteases which are insensitive or capable to degrade PIs. To explore contributory role of these bacterial communities, we earlier reported protease producing novel strain of *Bacillus* (*B. subtillis* RTSBA6 6.00) from gut of *H. armigera* (Shinde et al., 2012). In this study various strain of *Bacillus* identified were subjected to protease production in the nutrient broth supplemented with 1% skim milk protein. The highest protease activity was detected in all the isolates after 48 h of fermentation. The resulting extracts were tested for protease activity by dot blot assay using gelatin as substrate coated on X-ray film. A clear zone of gelatin hydrolysis was observed in each extracts suggesting protease producing potential of gut bacterial flora (Fig. 5).

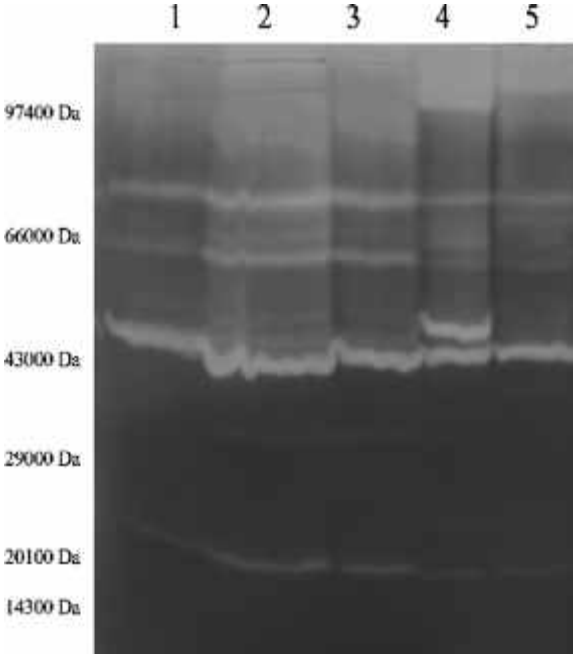

Fig. 6. In gel detection of protease activity (Non-denaturing 10% SDS-PAGE containing 0.1% gelatin). Lane 1: *Bacillus* sp. JR14, Lane 2: *Bacillus* sp. YP1, Lane 3: *Bacillus safensis* strain CG1, Lane 4: *Bacillus subtillis* strain KAVK2, Lane 5: *Bacillus megaterium* strain 47N.

3.3. Qualitative and quantitative analysis of bacterial proteases

The qualitative analysis of protease isoforms were assessed by zymography revealing several isoforms of proteases in each extracts with differing electrophoretic mobility (Fig. 6). The present finding is in agreement with previous study where isolated bacterial protease extracts showed twelve activity bands on zymographic gel (Shinde et al., 2012). On a gelatin-containing zymogram, a clear area of gelatin hydrolysis was formed in presence of protease, whereas the undigested gelatin stained blue. Generally all *Bacillus* species are well known as protease producer (Kim and Choi, 2000; Jeong and Han, 2001). The *Bacillus* species are aerobes or facultative anaerobe found in either free or symbiotic relationships (Turnbull, 1996). The expression of such enzymes by bacteria in the gut may allow the *H. armigera* to elude PIs based defense of host plants. Five bacterial strains namely *Bacillus subtilis*, *Bacillus cereus*, *Enterococcus gallinarum*, *Enterococcus mundtii*, *and Staphylococcus xylosus* with cysteine and serine like protease activity were isolated from the gut of velvet bean caterpillar and their possible involvement in adaptation to PIs is conferred (Visôtto et al., 2009b; Pilon et al., 2013). Besides, these bacterial proteases produced in gut are believed to involve in proteolytic processing of *B. thuringiensis* protoxin (Regode et al., 2016).

3.4. In vitro interaction study

In vitro interaction of bacterial proteases with host PIs were carried out selecting protease extracts produced by *Bacillus* sp. YP1, which was highest protease producer as assessed quantitatively

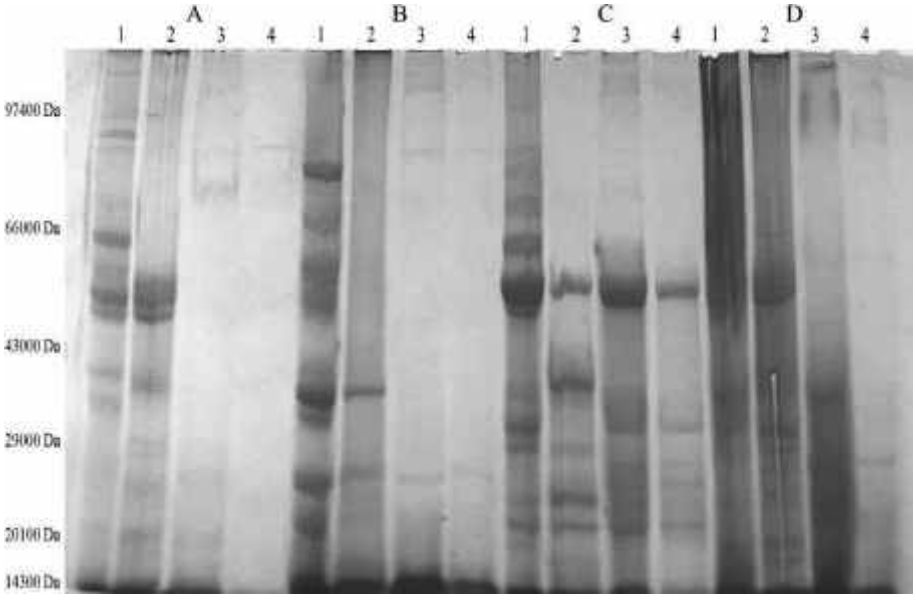

Fig. 7. Comparative digestion of host seed protein (100 μg) by equivalent volume of trypsin and protease from *Bacillus* sp. YP1 (non-denaturing 10% SDS PAGE). (A) Pigeonpea, (B) Chickpea, (C) Mungbean, (D) Soybean. Each lane 1: seed protein, lane 2: seed protein digested with trypsin (4 h), lane 3: seed protein digested with *Bacillus* protease (4 h), lane 4: seed protein digested with trypsin and *Bacillus* protease (4 h).

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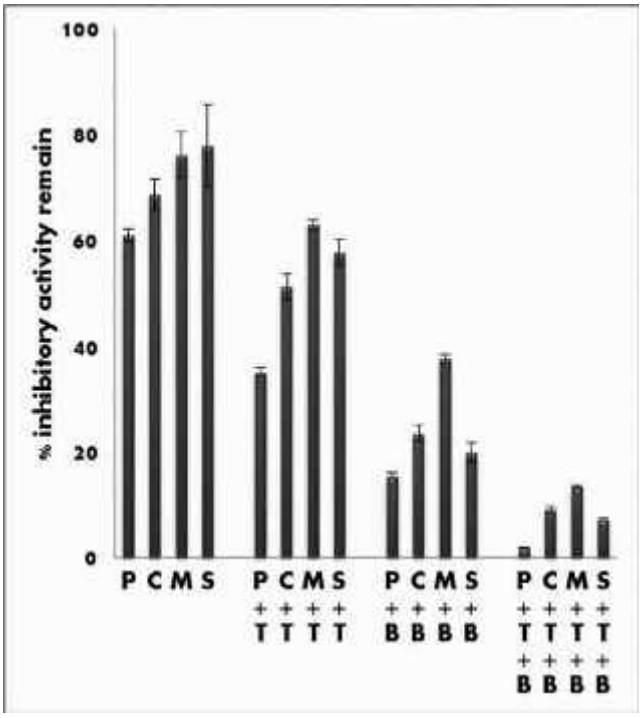

Fig. 8. Quantitative analysis of host PI activity after digestion. (P) Pigeonpea, (C) Chickpea, (M) Mungbean, (S) Soybean. Crude: PI activity from crude, Trypsin digest: PI activity from seed protein digested with trypsin (4 h), *Bacillus* digest: PI activity from seed protein digested with *Bacillus* protease (4 h), Trypsin + *Bacillus* digest: PI activity from seed protein digested with trypsin and *Bacillus* protease (4 h). Results are presented as mean ± SD, n = 3.

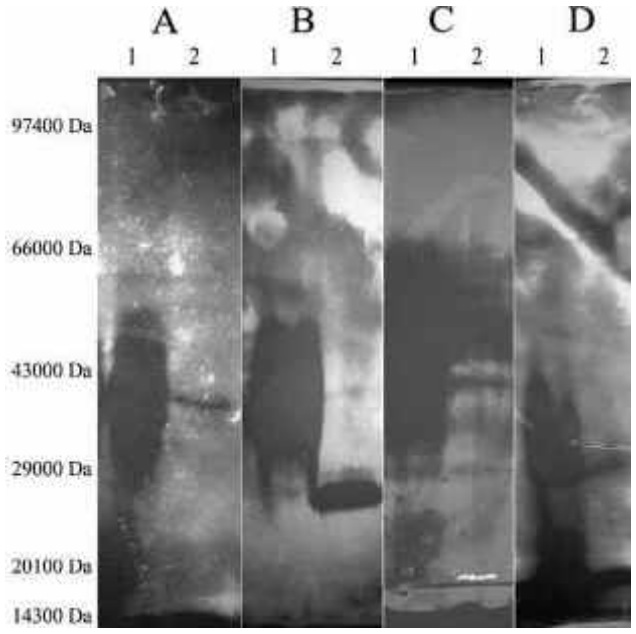

Fig. 10. Gel X-ray contact print (GXCP) analysis of remaining host PI activity after digestion by equivalent volume of trypsin and protease from *Bacillus* sp. YP1 (non-denaturing 10% SDS PAGE). (A) Pigeonpea, (B) Chickpea, (C) Mungbean, (D) Soyabean. Each lane 1: PI activity from seed protein digested with trypsin (4 h), lane 2: PI activity from seed protein digested with *Bacillus* protease (4 h).

by solution assay (data not shown). The seed proteins (100 μg) from pigeonpea, chickpea, mungbean and soybean were digested with equivalent concentration of trypsin (0.1 mg) and *Bacillus* sp. YP1 protease separately. About 100 μg of seed protein from all the hosts was also subjected to digestion using equivalent combination of selected protease mixture (Trypsin + *Bacillus* sp. YP1 protease). The results of digestion after 4 h were analyzed onto 10% SDS PAGE. The protein profiling of crude, trypsin digest and *Bacillus* sp. YP1 protease digest were obtained. Protein profile suggested that bacterial proteases have digested proteins from host seed which were resistant to trypsin (Fig. 7). The results also revealed that pigeonpea and chickpea proteins were found to be more susceptible to hydrolysis by *Bacillus* sp. YP1 protease as compared to mungbean and soybean. A greater digestion of seed proteins was observed by mixture of proteases (Trypsin + *Bacillus* sp. YP1 protease) as compared to trypsin and *Bacillus* sp. *YP1* proteases alone. Among seed proteins, the PIs activity of each host was reduced significantly with trypsin and *Bacillus* sp. YP1 mixture as compared to crude (Fig. 8). PIs of pigeonpea and chickpea were found to be more susceptible to trypsin and *Bacillus* sp. YP1 mixture digestion. This finding is in accordance with earlier reports where interactions between proteases of *H. armigera* and PIs of host plants studied

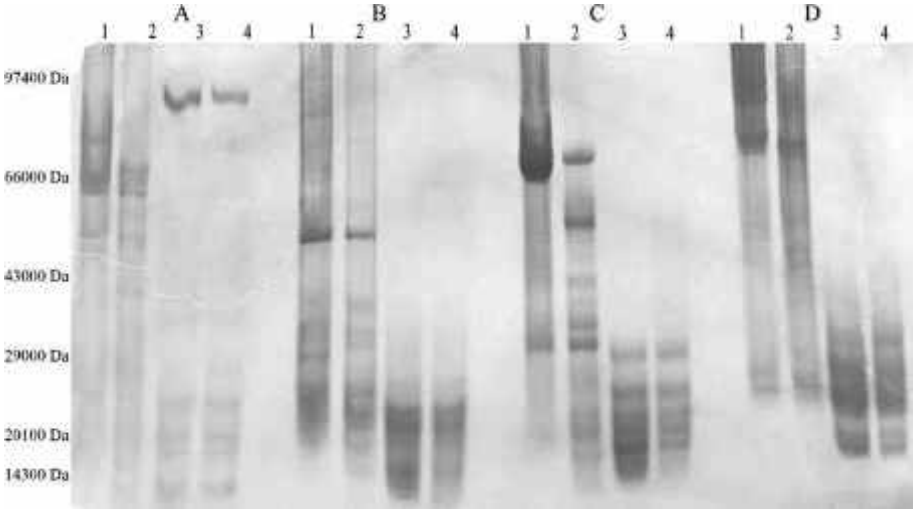

Fig. 9. Reverse zymographic analysis of host PI activity after digestion by equivalent volume of trypsin and protease from *Bacillus* sp. YP1 (10% SDS PAGE). (A) Pigeonpea, (B) Chickpea, (C) Mungbean, (D) Soybean. Each lane 1: PI activity from crude, lane 2: PI activity from seed protein digested with trypsin (4 h), lane 3: PI activity from seed protein digested with *Bacillus* protease (4 h), lane 4: PI activity from seed protein digested with trypsin and *Bacillus* protease (4 h).

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and explored (Giri et al., 2006; Patankar et al., 2001). Upon digestion by mixture of proteases (Trypsin + *Bacillus* sp. YP1 protease) only single PI of pigeonpea, chickpea and mungbean was found to resist proteolysis as assessed by reverse zymography and GXCP analysis (Figs. 9 and 10).

Above results demonstrated that bacterial communities associated with *H. armigera* gut has conserved in all tested host plants (Pigeonpea, Chickpea and Cotton) with slight variation after shift from one host to other suggesting conserved bacterial flora may involve in host insect survival in stress conditions such as exposure to host plant PIs. The bacterial communities with highest protease producing ability was identified and allowed to interact with host plant PIs in vitro. The results revealed that bacterial proteases have significant contribution in the degradation and detoxification of host seed protein rich in PIs. In nut shell, gut bacterial communities have significant impact in *H. armigera* digestive physiology and may be focused in detail to design competent strategies against infestation in field crops.

Author contribution

MSK and SPG are responsible for the main concept and final approval of the manuscript. MVP is responsible for data interpretation. AAS designed, performed, and coordinated the main study. FKS helped in manuscript writing and data analysis. PPG helped in sample collection and electrophoresis techniques.

Conflict of interest

The all authors of manuscripts declare that they there are no conflict of interests.

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