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A PROTEOMIC APPROACH TO IDENTIFY *BACILLUS SUBTILIS* INDUCED DEFENSE RELATED PROTEINS IN NONI CHALLENGED WITH *MELOIDOGYNE INCOGNITA*

Kavitha Govindasamy*[1], Nakkeeran Sevugapperumal[2] and Ahila Devi[3]

[1]Department of Tree Breeding, Forest College and Research Institute, Mettupalayam
[2]Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore
[3]Unit of Plant Pathology, Tamil Nadu Rice Research Institute, Aduthurai

ABSTRACT: Biopesticidal formulations of naturally occurring beneficial microorganisms are the most promising methods for more rational and safe crop management practices. *Bacillus subtilis* strains are important groups of natural antagonists that produce a broad spectrum of bioactive peptides with great potential for biocontrol of phytopathogens. Surfactin and iturin are antibiotic lipopeptides which imparts biocidal activity by direct suppression of plant-parasitic nematodes. Root knot nematode, *Meloidogyne incognita* is an economically important pathogen of noni, a herbal medicine gaining popularity in India. In this investigation, endophytic strains of *B. subtilis* were isolated from noni plants and tested for their nematicidal activity against root knot nematode, *in vitro*. The genomic DNA of the *Bacillus* strains was isolated and amplified by PCR to identify antibiotic genes *surfactin* and *iturin.* The strain Bs 5, with high surfactin and iturin activity was found to suppress egg hatching and caused juvenile mortality. Using the 'omic' approaches effort were taken to understand pathogenicity and defence-related genes and proteins expressed during the three way interaction of host, pathogen and biocontrol agent during disease development. Protein profiling was done using (2-DE) two-dimensional polyacrylamide gel electrophoresis and the differentially expressed proteins were analyzed by mass spectrometry. Up and down regulated protein spots were excised and analyzed by MALDI-TOF MS/MS, followed by cross-species protein identification. A total of 15 different proteins were found to be differentially expressed. Proteomic investigations revealed that certain functionally important defense related proteins *viz.*, Putative late blight resistance protein homolog, Toll-interleukin resistance domain containing protein, Translation Initiation factor IF1, Disease resistance protein putative Kalata-B1 and β-1,3-glucanase were induced by *B. subtilis* which are involved in the induction of defense response of host against the pathogen, *M. incognita.*
Key Words: *Bacillus subtilis,* Defense related proteins, Mass spectrometry, *Meloidogyne incognita*, Noni, Proteomics, 2D- PAGE.

*Corresponding author: Kavitha Govindasamy. Department of Tree Breeding, Forest College and Research Institute, Mettupalayam, India Email: kavitha_nem@yahoo.com

INTRODUCTION

Noni considered as a panacea to cure many diseases as a health supplement is gaining popularity for its use as an alternative herbal medicine. The root-knot nematode, *Meloidogyne incognita* (Kofoid and White) Chitw, is amongst the most damaging agricultural pest. Kavitha *et al*., [1,2] reported that yield loss caused by root knot nematode in noni was 46%.

One of the most commonly used and well-studied organisms, the rhizobacterium *B. subtilis*, has an average of 4–5% of its genome devoted to antibiotic synthesis and has the potential to produce more than two dozen structurally diverse antimicrobial compounds.

Endophytic bacteria *Bacillus subtilis* is a potential biocontrol agent used to manage root knot nematodes with characteristics of effective root colonization, multiple modes of action and promising ability to sporulate [3,4]. Cyclic lipopeptides of the surfactin, iturin and fengycin families are important metabolites produced by *Bacillus* species. The enzymes and antibiotics produced by *B. subtilis* (Ehrenberg) Cohn have been reported to have antagonistic effects on pathogenic microorganisms and induce systemic resistance against the plant pathogens by producing defense enzymes. The defense enzymes were reported to reinforce the cell wall structure and cause biochemical and physiological changes in the plant system, which are directly inhibitory to nematodes.

They impart successful biocontrol activity by direct suppression of phytopathogens and by reinforcing the host plant through stimulating the systemic resistance. It prevents the deleterious effects of a phytopathogenic organism and promotes plant growth through facilitating the uptake of nutrients from the environment. Several workers have reported biocontrol potential of *B. subtilis* against root knot nematode. [5, 6, 7,8,9,10] under *in vitro*, glass and field conditions.

Over the years, several studies have been performed to analyze plant–pathogen interactions. Recently, functional genomics strategies, including proteomics and transcriptomics, have contributed to the effort of defining gene and protein function and expression profiles. Using these 'omic' approaches, pathogenicity and defense-related genes and proteins expressed during phytopathogen infections have been identified and enormous datasets have been accumulated. Proteomics has dramatically evolved in the pursuit of large-scale functional assignment of candidate proteins and, by using this approach, several proteins expressed during phytopathogenic interactions. Molecular comparison of proteins isolated from target tissues i.e., roots of noni will give information on the biochemical/molecular changes associated during its interaction with root knot nematode. Applications of proteomic technology in crop protection are briefly explained by Kavitha and Nakkeeran, [2].

The present proteomic study was carried out to understand the molecular events induced in noni in terms of induction of defense related proteins upon treatment with the biocontrol agent *B. subtilis* strain BS5. To our understanding this is the first report of a discovery-based proteomics study of the interaction between a Heteroderidae nematode and its host, with emphasis on host plant resistant proteomes.

MATERIALS AND METHODS

Isolation of endophytic *B. subtilis* strains

Six antagonistic strains of *B. subtilis viz.*, Bs N 1, Bs N 3, Bs N 4, Bs N 7, Bs 5 and Bs N 11, were isolated from noni (*Morinda citrifolia* L.) plants collected in the Medicinal plants block of the Tamil Nadu Agricultural University, Coimbatore, India [11]. Healthy tissues of leaves and stems of noni plants were put in a beaker, soaked in distilled water and then drained. Tissues were rinsed in 70% ethanol for 30 seconds and then sterilized with a 0.1% $HgCl_2$ solution for 3 minutes. The tissues were then washed for ten times with sterile water [12]. Surface-disinfected tissues were aseptically macerated in homogenizers. The macerated tissues were diluted to 10^{-1} by adding nine volumes of sterile distilled water. Serial dilution was made up to 10^{-6} by taking 1 ml of well-shaken suspension and 9 ml water in tubes. One hundred µl from appropriate dilutions were spread plated on NA medium in sterile Petri plates.

Characterization of bacterial isolates

Preliminary identification of *B. subtilis* strains was done by doing colony characterization and biochemical tests *viz*., Gram staining, Catalase test, Anaerobic growth, Voges- Proskaeur test, Utilization of citrate, Growth on NaCl and Growth at 45°C and 4°C.

Detection of antibiotic genes of *Bacillus* isolate

To be sure that the culture filtrates of our strains of *B. subtilis* contained crude antibiotics, the genomic DNA from the *Bacillus* strains was isolated using the cetyl trimethyl ammonium bromide (CTAB) method described by Knapp and Chandlee [13] with slight modifications [14].

PCR amplification of antibiotic genes

Surfactin. The forward primer SUR3F (5' ACAGTATGGAGGCATGGTC 3') and reverse primer SUR3R (5' TTCCGCCACTTTTTCAGTTT 3') were used for amplification of *surfactin* gene (441 bp) (Ramarathnam, 2007). The 40 µl PCR reaction mixture contained DNA template 50 ng, 1xTaq buffer, 0.2 mM of each of dNTP mixture, 1 µM of each primers, 1.5 mM $MgCl_2$ and 2U of *Taq* DNA polymerase (Bangalore Genei, India). PCR amplification was performed in a thermocycler (Eppendorf Master cycler, German) using the following conditions: initial denaturation at 94 ℃ for 3 min, 40 cycles consisting of 94 ℃ for 1 min (denaturation), 57 ℃ for 1 min (annealing), 72 ℃ for 1 min (primer extension) and final extension at 72 ℃ for 10 min.

Iturin A. The forward primer ITUD1F (5' GATGCGATCTCCTTGGATGT 3') and reverse primer ITUD1R (5' ATCGTCATGTGCTGCTTGAG 3') were used for amplification of *iturin A* gene (647 bp) [15]. The 20 µl mixture contained approximately 50 ng of total DNA, 5 mM each dNTPs, 20 pmol of each forward primer and reverse primer and 0.5 U of *Taq* DNA polymerase (Bangalore Genei, India). PCR amplification was performed in a thermocycler (Eppendorf Master cycler, German) using the conditions: initial denaturation at 94 °C for 3 min, 40 cycles consisting of 94 °C for 1 min (denaturation), 60 °C for 1 min (annealing), 72 °C for 1 min (primer extension) and final extension at 72 °C for 10 min.

Agarose gel electrophoresis and Gel documentation

Agarose gel electrophoresis was performed based on the method given by Sambrook to check the quality of DNA and also to separate the products amplified through the polymerase chain reaction. After the separation with 1.5 % agarose gel at 50 UV, the PCR products were stained with ethidium bromide (0.5 µg/ml), photographed using Alpha imager TM1200 and analyzed using gel documentation system (Alpha Innotech Corporation, San Leandro, California).

***In vitro* bioassay**

Two *in vitro* bioassays were conducted simultaneously to assess the nematicidal activity of the crude antibiotic of the *B. subtilis* strain Bs 5.

***Hatching test* and mortality test:** Eggs masses of *M. incognita* were obtained from the infected roots of noni plants. One egg mass containing an average of 300-350 eggs was placed in a Syracuse dish and treated with 2 ml of crude antibiotic of Bs 5 at concentrations of 100, 50 and 25% and kept at 28 ± 1°C for different exposure times. 100 second stage juveniles were suspended in each Syracuse dish and incubated at 28 ± 1°C. Syracuse dishes without antibiotic, treated with distilled water served as control.

Proteomics study - Biological material

Two months old noni seedlings were imposed as *M. incognita* alone, *B. subtilis* strain BS5 alone, *M. incognita* + *B. subtilis* strain BS5 and Untreated control. Root tissues were collected at one month after treatment. Proteins extracted from the root samples were analyzed by two-dimensional polyacrylamide gel electrophoresis method to identify the proteins associated with short root phenotype [16,17]. Three biological replicates were collected and immediately transferred into liquid N and stored at -80°C until further use.

Protein Extraction

About 2 g root tissues from all the treatments were harvested and washed thoroughly with water to remove the soil particles adhering to it. Washed roots were dried and stored into liquid nitrogen at -80 ^{0}C. Frozen root tissues were ground in a mortar with liquid nitrogen and suspended in 10 % trichloracetic acid (TCA) in acetone with 0.07 % dl-dithiothreitol (DTT) at -20ºC for 1hr, followed by centrifugation for 15 min at 35,000 g. The pellets were washed once with ice cold acetone containing 0.07 % DTT at -20ºC for 1hr and centrifuged again for 15 min at 35,000 *g*. This washing step was repeated four to five times until the supernatant was clear (free of chlorophyll).

The final precipitated pellets were placed in a -80ºC freezer until frozen and then placed in an Lyophilizer in which the temperature gradient between the shelf (-40ºC) and the condenser (-78ºC) induced sublimation to remove moisture from the frozen pellets. The freeze-dry procedure was carried out under vacum overnight until the pellet become a dry powder. A total of 10 mg of the dried powder was dissolved in 350 µL of sample buffer containing 7 M urea, 2 M thiourea, 4 % 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.5 % ampholytes (Bio-Rad), and 0.7 % DTT (it is basic buffer but no one report this buffer's pH). The powder dissolved in the extraction buffer was gently shaken for 1 hr then centrifuged for 30 min at 35,000 g at room temperature. The supernatant was distributed in 100 µL aliquots and kept at -80ºC before 2D-PAGE analysis. Protein content was determined by Bradford assay (Bradford, 1976). About 100 mg of the protein was used for 2D-PAGE analysis [18].

Iso-electric Focusing

For analytical gels, the 17 cm IPG (Immobiline pH Gradient) strips (pH 4-7) were rehydrated overnight with 350 µl of rehydration buffer (8M Urea, 2% CHAPS, DTT (7 mg per 2.5 ml of rehydration buffer) and 0.5% (v/v) IPG buffer pH 4-7) containing the required quantity of proteins (100 µg) in a reswelling tray (Pharmacia Biotech., USA) at room temperature. The strips were allowed for about 12-14 hrs for rehydration and subjected to the first dimension separation. Isoelectric focusing (IEF) was carried out at 20ºC with a Pharmacia Multiphore II kit.

2D-PAGE

Equal amounts of protein (100 lg) from all the four samples were separated by Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). In the first dimension, IPG strips (BioRad Laboratories, USA) of 17-cm length and pH 4–7 were used. Electrophoresis was carried out at 400 V for 1 h, followed by 1000 V for 1 h and 2950 V for 24 h.

After IEF, the proteins were separated by SDS-PAGE in the second dimension using 13% polyacrylamide gels [18]. The gels were stained by the silver staining method. For each biological replicate, one set of gels with high resolution, run at different times, was selected for further analysis. The relative abundance of protein spots was quantified with Melanie III (GeneBio, Geneva, Switzerland), after silver staining the gels, and scanned with a densitometer (GS-700, Bio-Rad).

Protein Identification

Selected spots were excised from preparative gels (stained with Coomassie brilliant blue) (Mbeunkui *et al.,* 2010) and sent to the Shrimpex, Chennai for mass spectrometry. Mass spectra were obtained over a mass range of 1000–3,000 Da performed on a Voyager- DE STR. The proteins were identified by Mascot- Peptide Mass Fingerprint (www.matrixscience.com) database search. The following parameters were used for database searches: taxonomy, viridiplantae ; cleavage specificity, trypsin with 1 missed cleavages allowed; Peptide tolerance of 100 ppm for the fragment ions; allowed modifications, Cys Carbamidomethyl (fixed), oxidation of Met (variable). The peptide mass fingerprinting of the proteins were scored with the Mowse score.

RESULTS AND DISCUSSION

PCR amplification of antibiotic genes

The PCR amplified products, after separation in agarose gel electrophoresis followed by gel documentation, revealed the presence of *surfactin* and *iturin* genes in the *B. subtilis* strains. Biosynthetic gene specific primers SUR3F and SUR3R amplified a 440 bp of *surfactin* gene for BsN 3, Bs 5 and Bbv 57. Iturin specific primers amplified a fragment of 648 bp of *iturin* gene for BsN 4, Bs 5, Bbv 57 and BsN 11 (Fig.1 & 2).

***In vitro* bioassay**

The strain Bs 5, with *surfactin* and *iturin* genes, was found promising in reducing hatching of *M. incognita* eggs to a varying degree. The crude antibiotic extracted from the strain Bs 5 exerted lethal effects on eggs and juveniles of the root knot nematode. Egg hatching was hindered and only few juveniles emerged. A significant reduction in egg hatching (only 16.3 eggs hatched) was observed at 100% concentration after 72 h of exposure compared to the control (86.5 eggs). The crude antibiotic had lethal effect also on the juveniles of the nematode as shown by the reduced movement and increased death. The reduction in the movement was irreversible and the death of the juveniles was confirmed when they were transferred to distilled water for 24 hrs. The greatest juvenile mortality (92.3 J_2) was recorded in the 100% concentration of the crude antibiotic after 72 h of exposure (Table 1).

2-D PAGE analysis

The differentially expressed proteins were categorized into nine different classes based on the pattern of differential expression. Protein spot N2 was up regulated only in control, N1 and N15 were down regulated in *M. incognita* alone, N4, N10 and N12 were up regulated in *M. incognita* alone, N6 was protein newly present in *M. incognita* alone, N7, N8 and N9 were up regulated in *M. incognita* alone and interaction, N3 was up regulated in *M. incognita* alone and down regulated in *B. subtilis* alone, N5 was up regulated in *M. incognita* alone and completely absent in *B. subtilis* alone, N13 and N14 were down regulated in both control and *M. incognita* alone but up regulated in both interaction and *B. subtilis* alone, N11 was absent in control and PGPR alone but newly present in *M. incognita* alone and interaction (Tables 2&3, Fig 3).

Of these we have chosen 2-DE gel with pH 4-7 range and a 12% linear polyacrylamide gel for our experiments. Most distinct nine differential spots were sequenced and functionally characterized. Analysis of PMF (Probability Mass Function) data of nine proteins derived by MS analysis using MASCOT search algorithm showed homology to the following proteins 1) Putative late blight resistance protein homolog 2).

Toll-interleukin resistance domain containing protein 3) Glutathion peroxidase *(Dimocarpus longer)* 4) Translation Initiation factor IF1, chloroplastic 5) Disease resistance protein putative (A.t) 6) Kalata- B1-OS *Oldenlandia officinalis* and 7) Cellulose synthase 2 (*Linum usitatisimum)* 8) Photosystem II subunit H partial (chloroplast) 9) DIMBOA-UDP glucosyl transferase *Zea mays* (Table 2).

Table 1. Effect of crude antibiotic of *B. subtilis* strain Bs 5 on hatching of eggs and mortality of second stage juveniles of *Meloidogyne incognita*

Crude antibiotic concentration (%)	Exposure time (hrs)					
	No. of eggs hatched/egg mass			No. of juveniles dead /100 juveniles		
	24	48	72	24	48	72
100	7.85 (2.78)	12.63 (1.61)	16.30 (4.02)	60.58 (1.78)	70.23 (1.84)	92.58 (1.96)
50	12.72 (3.37)	29.02 (5.45)	36.54 (6.03)	38.20 (1.88)	48.11 (1.68)	62.44 (1.78)
25	34.13 (5.83)	38.22 (6.18)	42.39 (6.51)	30.13 (1.47)	40.73 (1.60)	46.46 (1.66)
Control (Distilled water)	75.45 (8.78)	167.21 (12.93)	286.53 (6.92)	0.0 (0.28)	0.0 (0.28)	4.01 (0.64)
CD (0.05)	0.3712	0.2740	0.1802	0.0400	0.0334	0.5467

Figures in parenthesis are square root transformed values
*Figures in parenthesis are arcsine transformed values
Pooled analysis of data collected from two sets of experiments

Table 2. Differential proteome analysis of noni root during interation with root knot nematode and *B. subtilis* strain BS5

S.No	Spot ID	Change in root protein expression
1.	N2	Up regulated in control
2.	N15	Up regulated in interaction alone
3.	N10	Up regulated in *M. incognita* alone
4.	N12	Up regulated in *M. incognita* alone
5.	N1	Down regulated in control
6.	N4	Down regulated in control, up regulated in interaction
7.	N6	Newly present in *M. incognita* alone
8.	N7	Up regulated in *M. incognita* alone and interaction
9.	N8	Up regulated in *M. incognita* alone and interaction
10.	N9	Up regulated in *M. incognita* alone and interaction
11.	N3	Up regulated in *M. incognita* alone and down regulated in PGPR alone
12.	N5	Up regulated in *M. incognita* alone and completely absent in PGPR alone
13.	N13	Down regulated in both control and *M. incognita* alone but up regulated in both interaction and PGPR alone
14.	N14	Down regulated in both control and *M. incognita* alone but up regulated in both interaction and PGPR alone
15.	N11	Absent in Control and PGPR alone but newly present in *M. incognita* alone and interaction

Table 3. Differential expression of noni root proteins during root knot nematode and PGPE interaction identified by MALDI/TOF & Peptide Mass Fingerprinting (PMF)/ MS/MS analysis

S. No	Protein (Species)	Calculated MW/PI	Expected MW/PI	Significance Score	E-value	Sequence coverage %	Accession number	Protein ID	Peptide matches	Remarks
1.	Toll-interleukin resistance domain containing protein	43563/9.11	22.5/ 4.5	40/73	1.3 e+02	40	NP177434	N4	8	Defense signaling pathway, innate immune response
2.	Kalata- B1-OS	13718/7.51	57/4.5	45/58	1	85	P56254	N7	5	Cyclotoid with Potent insecticidal activity
3.	Glutathion peroxidase *(Dimocarpus longer)*	19222/9.18	57.5/4.2	63/73	0.54	55	AFF18778	N8	9	Protect organism from oxidative damage
4.	Putative late blight resistance protein homolog *Solanum demicium*	156612/5.9	28/6.8	44/58	1.3	17	Q6L3Z4	N13	19	Resistance protein
5.	Cellulose synthase 2 (*Linum usitatisimum)*	17138/9.33	26/6.6	46/73	29	60	ABN09210	N14	5	B 1,4- liked glucose residues, defense mechanism
6.	Lichenase II precursor Putative (O.s group)	16042/4.77	22.5/4.9	45/73	38	81	ABF95906	N3	4	Cellulose, glucan, 1,3-β glucosidase
7.	DIMBOA-UDP glucosyl transferase *Zea mays*	49895/5.44	44/6.7	43/58	2.7	29	Q8W2B7	N11	9	Glycosylation-production of low molecular weight secondary metabolites
8.	Translation Initiation factor IF1, chloroplastic	9371/10.27	58/ 4.5	43/58	1.5	65	Q85F18	N6	5	Stimulates *invivo* initiation of protein synthesis
9.	Photosystem II subunit H partial (chloroplast)	3087/5.1	57.5/10 .75	54/73	5.1	100	AAQ18521	N9	4	Oxygenic photosynthesis

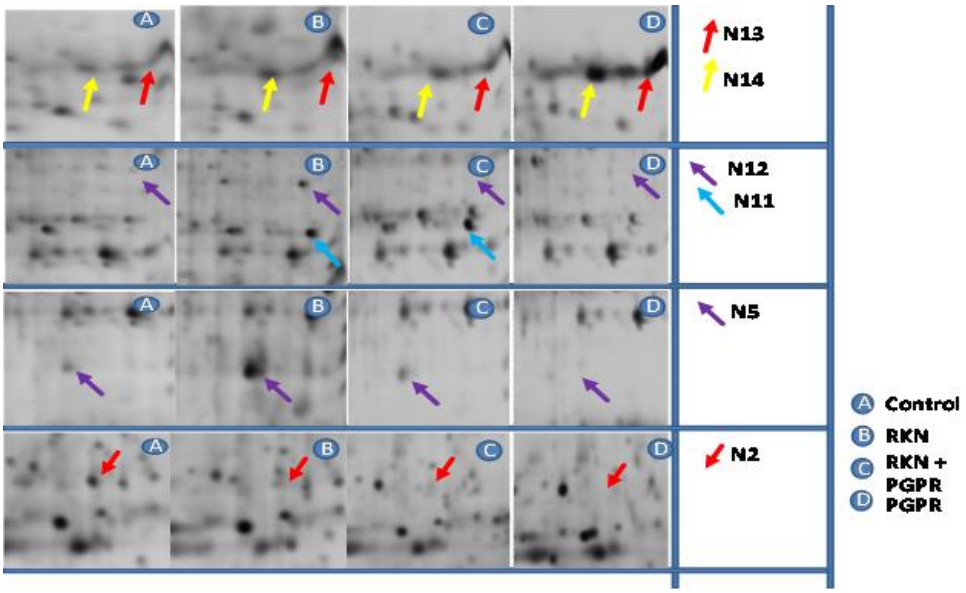

Fig 1. Comparison of protein spots of 2D-PAGE analysis

The analysis of the present study revealed that the protein spots induced by root knot nematode *viz*., N6 and N9 has role in initiation of protein synthesis and oxygenic photosynthesis respectively. New protein spot N11 induced in root knot nematode challenged noni is involved in production of low molecular weight secondary metabolites which might be a resistant response of noni plant against the nematode. Whereas protein spots induced in the *B. subtilis* treatment *viz*., N4 is involved in defense signalling pathway, N7 is a Cyclotoid with potent insecticidal activity, N13 is a putative late blight resistance protein homologue and N14 is β-1,3-glucanase residues which induce the defense mechanism and impart strength against pathogens..

With the 2-DE proteomic approach coupled with MS/MS mass spectrometry, this is the first study to monitor protein expression patterns in noni response to *B. subtilis.* In this study, we detected proteins involved in various metabolic pathways were likely involved in the host plant's defense system in noni roots challenged with root knot nematode and biocontrol agent. Some defense related proteins were increased in plants treated with *B. subtilis* alone whereas additional defense related proteins were increased in plants treated with both *B. subtilis* and root knot nematode in a quantitative manner. Essential role of proteomics in understanding the biology of plants, a global plant proteomics organization is established to properly organize, preserve and disseminate collected information on plant proteomics (Ganesh *et al*., 2011). Utilizing the outcome of proteomics study and identification of such proteins will provide improved knowledge on the molecular basis of developing resistant variety of noni against root knot nematode and other soil pathogens.

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