International Journal of Research in BioSciences
Vol. 6 Issue 4, pp. (59-70), October 2017
Available online at http://www.ijrbs.in
ISSN 2319-2844

Research Paper

Accumulation of transcripts of defense enzymes involved during rhizobacteria mediated induced resistance against pearl millet downy mildew disease

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(Received August 20, 2017, Accepted September 15, 2017)

Abstract

Plant growth promoting rhizobacteria strain *Bacillus pumilus* INR 7 induced systemic and durable resistance against downy mildew disease of pearl millet caused by the oomycete *Sclerospora graminicola*. The underlying mechanism of this induced resistance was studied by analyzing the pattern of transcript accumulation of some of the important defense enzymes in the *B. pumilus* INR 7 treated seedlings in comparison to the downy mildew resistant and susceptible seedlings. Accumulation of mRNA for defense enzymes like glucanase, chitinase, phenylalanine ammonia lyase, peroxidase and polyphenol oxidase, gene was measured in response to *B. pumilus* INR 7 treatments and the same was compared with the resistant and susceptible seedlings. The time course of enzyme expression correlated with the earlier report that, in an incompatible interaction, it is the speed and intensity of the defense reaction that determine the resistance to the pathogen. For most of the enzymes examined, the highest levels of transcripts were observed in resistant seedlings followed by induced resistant seedlings in comparison with the susceptible seedlings. For each enzyme, very low levels of mRNA were present in susceptible seedlings. Inducer treatment caused an increase in transcript accumulation, but generally not to the same degree as resistant seedlings. PAL and PPO transcripts were expressed in highest levels in the induced resistant seedlings implicating a major role in *B. pumilus* induced resistance against pearl millet downy mildew disease.

Keywords: transcript accumulation, defense enzymes, pearl millet, downy mildew, rhizobacteria induced resistance

Introduction

Plants interact with beneficial microorganisms like plant growth promoting rhizobacteria (PGPR) that suppress diseases and enhance plant growth. In recent years, use of non-pathogenic saprophytic PGPR as an inducer of systemic resistance in crop plants against different pathogens has been well demonstrated[1]. However, there is a significant gap in our understanding of molecular biology of most interactions of plants with PGPR that lead to induced disease resistance. Plants are endowed with various defense related genes and they express a variety of defense products in response to pathogen attack as a consequence of transcriptional activation. Accumulation of defense gene transcripts generally commences within minutes to hours around the infection sites and several hours

or days later at distant sites over the whole plant. During local and systemic responses of plants, a large group of defense enzymes, PR proteins and signal molecules are synthesized in high amounts to display a broad spectrum of antimicrobial activity. Through intensive studies, numerous genes involved in these processes have been identified[2,3], but, the transcript accumulation pattern of defense enzymes during PGPR mediated induced resistance has not received much attention. Defense response genes are induced in both incompatible and compatible plant-pathogen interactions. However, mRNA accumulation for many plant defense genes is more rapid during interactions involving a plant expressing resistance to a particular pathogen[4,5]. Therefore, studying the defense responses at the molecular level has become increasingly important form various perspectives, most important being devising effective control methods by genetic engineering.

A growing body of evidence from various studies indicates that increased resistance of plants may be associated in part with marked metabolic changes in host, particularly the accumulation of mRNA transcripts which result in the enhanced production of peroxidases polyphenol oxidases, and phenolic compounds, accumulation of hydrolases, such as chitinases and β-1,3-glucanases with antimicrobial potential; and deposition of structural polymers, such as lignin and hydroxyproline-rich glycoproteins [6,3]. If one considers that the increased production of enzymes and signal compounds may be of key importance in the resistance process, then studying the speed and magnitude of the accumulation of transcripts of these substances is very vital for devising strategies for pathogen control.

Earlier we have demonstrated that plant growth promoting rhizobacteria strain *Bacillus pumilus INR 7* effectively induces systemic and stable resistance against pearl millet downy mildew disease [7]. The present study was undertaken to elucidate the mechanism of induction of resistance by *Bacillus pumilus* INR 7 against pearl millet downy mildew disease at the molecular level by studying the pattern of accumulation of transcripts of some important defense enzymes that are said to have a role in the induction of resistance.

Host

Seeds of pearl millet cultivars HB3 and IP18292 which are highly susceptible and resistant to the downy mildew pathogen *S. graminicola* respectively were obtained from the International Crop Research Institute for Semi Arid Tropics (ICRISAT), Hyderabad, India and All India Co-ordinated Pearl Millet Improvement Project, Mandor, Rajasthan, India.

Source of pathogen and inoculum preparation

Sclerospora graminicola isolated from pearl millet cv. HB3 grown under field conditions heavily infested with oospores of the pathogen was used. The pathogen was maintained on its susceptible host prior to use [8]. Leaves of pearl millet showing profuse sporulation of *S. graminicola* on the abaxial side were collected in the evening from the plants maintained under greenhouse conditions. Collected leaves were thoroughly washed under running tap water to remove previous crop of sporangia. The leaves were then blotted dry, cut into small pieces, and kept in a moist chamber for sporulation. The next morning, the fresh crop of sporangia was harvested into distilled water. For use as inoculum, the zoospore concentration was adjusted to 40,000/ml using a haemocytometer.

PGPR strains and inoculum preparation

PGPR strain *Bacillus pumilus* INR-7 was obtained from the culture collection of the Department of Entomology and Plant Pathology, Auburn University, USA (Courtesy: Prof. J.W. Kloepper and Prof. M.S Reddy). *B. pumilus* INR-7 was stored in tryptic soy broth (TSB) amended with 20% glycerol at -80^0C prior to use. Bacterial cell suspensions were prepared by streaking PGPR strain from ultra-cold storage onto tryptic soy agar (TSA), incubating at 27^0C for 24 h to check for purity, and then transferring single colonies to TSA. After 24 h, the bacterial cells were harvested from the TSA plates in sterile distilled water (SDW) and centrifuged at 6000 rpm for 5 min. The pellet obtained was resuspended in SDW. The optical density of the suspension was adjusted using a UV visible spectrophotometer (Hitachi U-2000, Japan) following the method of Mortensen[9] to obtain a final density of 10^8 cfu/ml.

Inducer treatment

Bacillus pumilus INR-7 was used as seed treatment. The seeds of pearl millet cv. HB3 were surface sterilized with 0.02% mercuric chloride for 5 min and rinsed thoroughly in SDW. Bacterization of the seeds was achieved by soaking seeds in *Bacillus pumilus* INR-7 (5 g/25 ml), prepared as described earlier and amended with 0.2% sterilized carboxymethyl cellulose (CMC) as a sticker. The

suspensions were incubated at 26^{0}C in a rotary shaker for 6 h to facilitate attachment of bacterial cells to the seed coat. Later, the seeds were allowed to dry in an incubator at 30^{0}C. Seeds treated with SWD amended with CMC served as nonbacterized control.

Inoculation and sampling

Seeds were germinated on discs of moist blotter paper in Petri dishes at 25±2◦C for 2 d. *S. graminicola* was maintained on its susceptible host (HB3 genotype of pearl millet) under greenhouse conditions. Two-day-old seedlings of resistant (IP18292), susceptible (HB3) and induced susceptible (HB3) treated were root-dip inoculated with a zoospore suspension of 4 x 10^4 ml^{-1}, and incubated in dark at 25±1^{0}C. Seedlings were harvested at 0, 3, 6, 9, 12, 24, 48 and 72 h after inoculation and immediately wrapped in aluminum foil and stored at -70^{0}C until further use.

cDNA Probes used

Heterologous cDNA probes from barley for Peroxidase (pBH 6-301 (1284 bp), glucanase - (pBH 72-I1) were kindly gifted by Dr. Nandini Prasad Shetty, Assistant Professor, KVL, Denmark. Phenylalanine Ammonia Lyase (*At PAL* (6 kb gene clone from *Arabidopsis thaliana*, AtPAL gifted by C. Lamb, Salk Institute for Biological Sciences, San Diego, USA), chitinase (CH 11. 1 kb gene probe from maize gifted by J.M. Widholm, University of Illinois, Urbana-Chormpagne Urbana, USA) and Polyphenol oxidase probe from wheat, a gift from Dr. James V. Andersen, USDA-ARS-NPA, Fargo, ND, USA.

Plasmid DNA Extraction

A single recombinant colony from the master plate was incubated in 1 ml LB medium containing ampicillin (100 μg ml^{-1}). This was grown to saturation at 37^{0}C overnight at 175 rpm and the culture was pelleted in a 1.5 ml Eppendorf tube. The pellet was suspended in 200 μl of solution A (4 mg lysozyme ml^{-1} of GTE solution), 300 μl of solution B (1.0% SDS and 0.2 N NaOH) and 300 μl of solution C (7.5 M ammonium acetate) were added, the contents were mixed well and kept on ice for 10 minutes. The solution was centrifuged for 10 min at 10000 rpm to pellet the chromosomal DNA and cell debris. The plasmid DNA from the supernatant was precipitated with 2.5 volumes of 95 % Ethanol for 10 min on ice. This was followed by a spin at 10,000 rpm at 40C for 10 min, to pellet plasmid DNA and RNA. The pellet, washed with 70 % ethanol and dried under vacuum, was dissolved in $T_{10}E_1$ buffer. The plasmid was treated with 1 μ (10mg/ml) of RNase (DNase free) at 37^{0}C for one hour to remove RNA. It was followed by phenol: choloroform treatments to remove RNase.

Using PCR amplification for insert purification

Alternatively, PCR was used for amplification and then inserts were purified using Sephadex® 6-50 or Spin Column Elutips® or similar size exclusion media. In this method, the extracted plasmid DNA was diluted 100 times by mixing 1 μL plasmid DNA in 99 μL of water and 5 μL of this was used in a PCR reaction using M13 forward and M13 reversal universal primers. The following recipe was used to make the PCR reaction:

PCR components		**PCR conditions**		
Water	32.5 μl	94°C	1 minute	33 cycles
10X PCR buffer	5.0 μl	40°C	1 minute	
2.5 mM dNTP	2.0 μl	70°C	2 minutes	
Universal primer	1.0 μl	72°C	5 minutes	
Reversal primer	1.0 μl	4°C	Storage	
Taq polymerase	0.5 μl			
Template DNA	5.0 μl			
Total	50.0 μl			

Labeling of probes

The random-primed method of Feinberg and Vogelstein[10] was used for labeling DNA with α-^{32}P. The double stranded DNA was denatured, and complementary labelled fragments were synthesized from octadeoxinucleotides primers of random sequence. By including a nucleotide triphosphate with a proximal (α)^{32}P-labelled phosphate in the reaction mixture, the DNA polymerase will produce

radioactively labelled DNA. Probe labelling was carried out according to NE Blot® kit (New England Biolab Inc. USA) by initially denaturing the probe at 95^0C for 10 min, then placed on ice for 5 min followed by incubating in a reaction mixture consisting of Primer and buffer 5 µl, dNTPs 6 µl, ^{32}P dATP 4 µl and Klenow 1.5 µl at 37^0C for 1 h. The reaction was stopped by adding 400 µl of 200 mM EDTA and denatured at 95 ^{0}C for 5 min followed by chilling on ice for 10 min prior to hybridization.

RNA Isolation

The success of differential gene expression studies depends on the integrity of the RNA and on it being free of chromosomal DNA contamination. The prime concern in isolating high quality RNA are usually attributed to the presence of ribonucleases (RNAses) and a high content of secondary metabolites such as phenolics and polysaccharides, which bind to RNA upon cell lysis resulting in extensive degradation and low yields. To avoid RNAse contamination during RNA extraction a set of protocol was followed which is described as follows:

Glasswares were treated with 0.1% diethylpyrocarbonate (DEPC) water, autoclaved and baked at 160^0C overnight to inhibit RNase activity. All the reagents were prepared with DEPC treated water. Frozen plant material were ground to a fine powder in liquid nitrogen, to that 10 ml of extraction buffer containing 150 mM LiCl, 5 mM EDTA pH 8.0, 50 mM Tris-HCl pH 9.0, 5 % SDS, and 10 ml of phenol:chloroform (1:1) was added. The tube was shaken thoroughly for 10 min until an emulsion was obtained. The tubes were then centrifuged at 2000 rpm for 15 min at room temperature. Supernatant was transferred to a new centrifuge tube containing 10 ml phenol: chloroform, shaken vigorously and centrifuged at 2000 rpm for 15 min at room temperature. This step was repeated twice. The supernatant obtained was transferred to a centrifuge tube containing 10 ml of chloroform, shaken well and centrifuged at 2000 rpm, 10 min at room temperature. Upper phase was transferred to a sterile corex tube, add $1/10^{th}$ volume of 3 M NaOAc (pH 5.5) and 2.5 volumes of 96% ethanol were added and stored at -20^0C overnight. The tubes were centrifuged at 10,000 rpm at 4^0C for 30 min. Pellet was dissolved in 0.15 M NaOAc and to that 6 ml of ice cold 4 M NaOAc was added and kept in -20^0C overnight. The tubes were centrifuged at 10,000 rpm at 4^0C for 30 min. Supernatant was drained off and the pellet was resuspended in 1.0 ml 3 M NaOAc and transferred immediately onto 1.5 ml Eppendorf tubes on ice for 1 h. It was centrifuged at 10,000 rpm, 30 min at 4^0C, supernatant drained off, 0.5 ml 70% ethanol was added to the pellet and vortexed briefly. The tubes were then centrifuged at 10,000 rpm, 30 min at 4^0C. Pellet was dried for 10 min and dissolved in 50 µl of sterile water and stored in freezer (-20^0C). Purity of RNA was checked by taking absorbance ratio of 260 and 280 NM and integrity of the RNA sample was checked by electrophoresing on denaturing 1.2% formaldehyde agarose gel.

Northern blotting

Approximately 30 µg of total RNA sample along with loading buffer was kept in boiling water bath for 2 min, chilled on ice for 5 min and electrophoresed on 1.2% agarose gels containing 7.4% formaldehyde, 1x MOPS buffer, 5 mM sodium acetate and 1mM EDTA. After electrophoresis, RNA gel was then shaken in excess water at room temperature for 2 X 15 minutes in order to remove formaldehyde. RNA was then transferred on to Hybond N membranes (Amersham, UK) by capillary transfer using 20 X SSC overnight. After blotting the membrane was washed in 2x SSC at room temperature for 2 X 10 min and exposed to UV- for crosslinking (UV-Stratalinker ^{TM} 1800, USA). The nylon membrane was later baked at 80^0C for 1 ½ hr.

Northern hybridization

Pre-hybridization: Northern blots were pre-hybridized at 42°C with 10 ml of pre-hybridization solution (0.8 ml of 100X of Denhardt's solution, 0.5 ml of 10 % SDS, 2.0 ml of 20X SSC, 20 µl of 0.5 M EDTA, 0.2 ml of 10 mg/ml salmon sperm DNA and sterile distilled water to 6.5 ml) for 4 h in case of new blots and 1 h for stripped blots. Pre-hybridization was performed in a Techne Hybridizer (HB-1D).

Hybridization

Labeled probe was added to the hybridization bottles containing blots and pre-hybridization mixture and incubated at 42°C in hybridization oven for at least 16 h. Care was taken to remove air bubbles present between the blot and the hybridization bottle.

Washing of blots

Following hybridization, the blots were washed using four changes of 50 ml each of ^{32}P-wash solution. Each wash was carried out for 15 min at 42°C in hybridization bottles using the hybridization oven.

The first two washes were done using wash 1 solution (2X SSC and 0.1 % SDS) followed by two washes with wash 2 solution (0.1 X SSC and 0.1 % SDS). Membranes were air dried and enclosed in cling films.

Autoradiography
Autoradiography was carried out at -70°C by exposing the membrane to Phosphor Image analyzer (FLA 5000, Fuji Film, Japan) using red lazer. Analysis of transcript intensity was carried out using the Bioprofile image analysis system (Vilber Lourmat, France).

Results and Discussion

Glucanase
Constitutive level of glucanase transcripts was a 7.2. folds higher in resistant seedlings in comparison to susceptible seedlings. In resistant seedlings glucanase accumulation was detected as early as 0 h and its accumulation steadily increased up to 48 hpi and decreased at 72 hpi. Maximum glucanase was observed at 24-48 h post inoculation. In induced resistant seedlings glucanase accumulation started from 0 h post inoculation and gradually increased (Figure 1). However, the intensity was lower and maximum glucanase transcripts was noticed at 12 and 24 h post inoculation. In susceptible seedlings glucanase accumulation started from 6 h onwards and maximum was observed at 12 and 24 h post inoculation. At 12 h post inoculation glucanase in resistant and induced resistant seedlings was 8.1 and 5.6 folds higher than susceptible seedlings. At 24 h post inoculation resistant and induced resistant seedlings had 8.8 and 4.9 folds higher glucanase expression in comparison to susceptible seedlings.

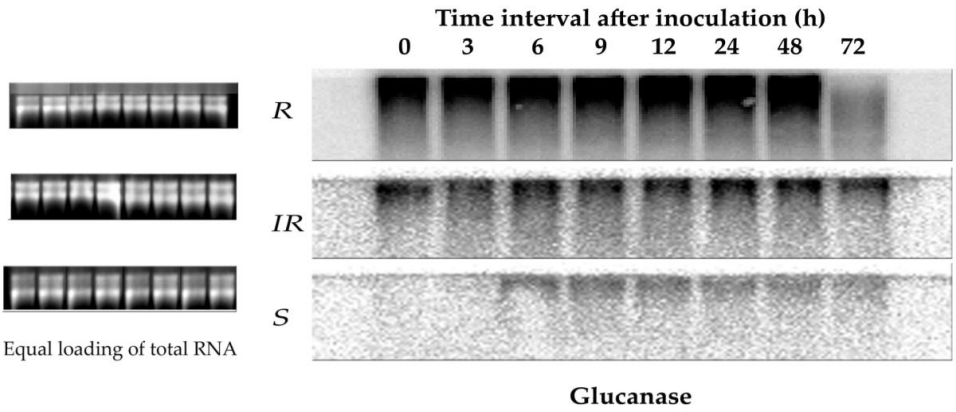

Figure 1: Northern blot analysis showing the temporal pattern of accumulation of glucanase in resistant (R), induced resistant (IR – treated with *B. pumilus INR7*) and susceptible (S) pearl millet seedlings after inoculation with *S. graminicola*

Chitinase
Chitinase mRNA expression exhibited strikingly exclusive expression patterns following inoculation wherein resistant and induced resistant seedlings showed biphasic accumulation of chitinase transcripts. In resistant seedlings chitinase accumulation started from 0 h and increased at 3 h which again decreased at 6 h and again started increasing from 9 h onwards. Maximum accumulation was at 24 h post inoculation. Similarly, in induced resistant seedlings followed the same pattern of accumulation of chitinase m RNA, but the intensity was significantly lower. At 6 and 48 h chitinase activity was 1.2 and 3.6 folds higher in resistant seedlings compared to induced resistant seedlings. Contrarily in susceptible seedlings chitinase accumulation was noticed 9 h onwards which was maximum at 24 h and decreased thereafter. Constitutive level of chitinase was detectable only in resistant seedlings (Figure 2).

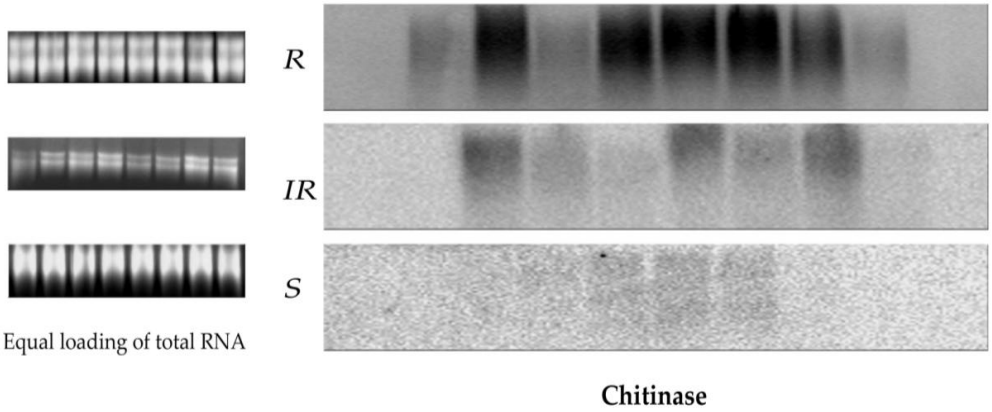

Figure 2: Northern blot analysis showing the temporal pattern of accumulation of chitinase in resistant (R), induced resistant (IR – treated with *B. pumilus INR7*) and susceptible (S) pearl millet seedlings after inoculation with *S. graminicola*

Phenylalanine ammonia lyase

No constitutive level of PAL was detected in all the categories of seedlings. Detectable levels of PAL were observed in resistant seedlings at 6 h post inoculation and it increased at 9 h which again remained steady up to 24 h and again increased at 48-72 h post inoculation. In induced resistant seedlings PAL expression started from 6 h onwards and increased up to 24-48 h post inoculation and decreased thereafter. Contrastingly in susceptible seedlings PAL accumulation was observed only 6 h at very low levels and gradually increased at 24 and 48 h and again decreased thereafter. The PAL transcript accumulation was highest in the induced resistant seedlings at all time points compared to the resistant and susceptible seedlings. In resistant seedlings maximum PAL accumulation was noticed at 48 hpi which was 11.3 folds higher than the susceptible seedlings at the same time interval. In induced resistant seedlings PAL accumulation was highest at 48 hpi and was 0.8 and 12.6 folds higher than the resistant and susceptible seedlings at the same hour (Figure 3).

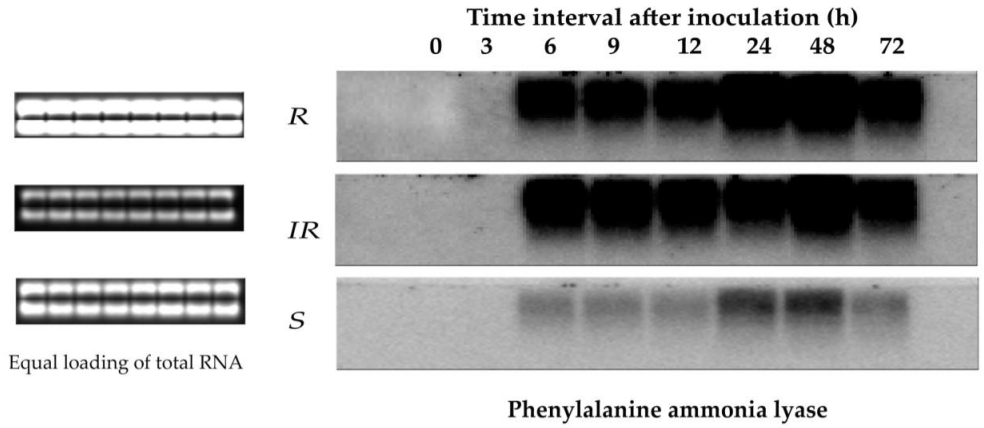

Figure 3: Northern blot analysis showing the temporal pattern of accumulation of phenylalanine ammonia lyase in resistant (R), induced resistant (IR – treated with *B. pumilus INR7*) and susceptible (S) pearl millet seedlings after inoculation with *S. graminicola*

Peroxidase

Strong signals of accumulation of peroxidase transcripts were detected at all time intervals in resistant, induced resistant and susceptible seedlings. However, the intensity was higher in resistant and induced resistant seedlings compared to susceptible seedlings. At 0 h interval peroxidase transcript accumulation was 1.7 and 1.2 folds higher in resistant and induced resistant seedlings compared to susceptible seedlings. At 3 h post inoculation peroxidase was 5.4 and 5.1 folds higher in resistant and induced resistant seedlings than susceptible seedlings. In induced resistant seedlings maximum peroxidase was accumulated at 24 – 48 h post inoculation whereas in resistant maximum accumulation was noticed at 48 - 72 h post inoculation (Figure 4).

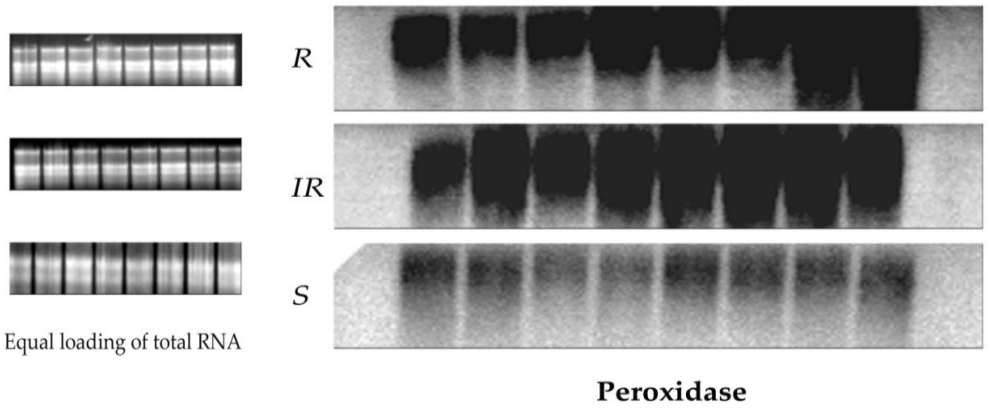

Figure 4: Northern blot analysis showing the temporal pattern of accumulation of peroxidase in resistant (R), induced resistant (IR – treated with *B. pumilus INR7*) and susceptible (S) pearl millet seedlings after inoculation with *S. graminicola*

Polyphenol Oxidase

Temporal expression of transcript accumulation was tested for the enzyme polyphenol oxidase after pathogen inoculation in resistant, induced resistant and susceptible pearl millet seedlings (Figure 5). The PPO transcripts were present to some degree even in the constitutive levels in all categories of seedlings. However, following pathogen inoculation, there was a marked difference in the transcript accumulation in resistant, induced resistant and susceptible seedlings. The intensity of PPO transcript accumulation was very high in induced resistant seedlings followed by resistant seedlings. There was very low transcript accumulation in susceptible seedlings. In induced resistant seedlings transcript accumulation started at 3 hpi and reached a maximum of at 12-24 hpi. At 12 hpi the transcript accumulation of PPO was 13.1 and 6 folds more in induced resistant seedlings compared to the resistant and susceptible seedlings. In resistant seedlings maximum PPO transcript accumulation was noticed at 24 hpi, which was 7.1 folds higher than the control at this time point.

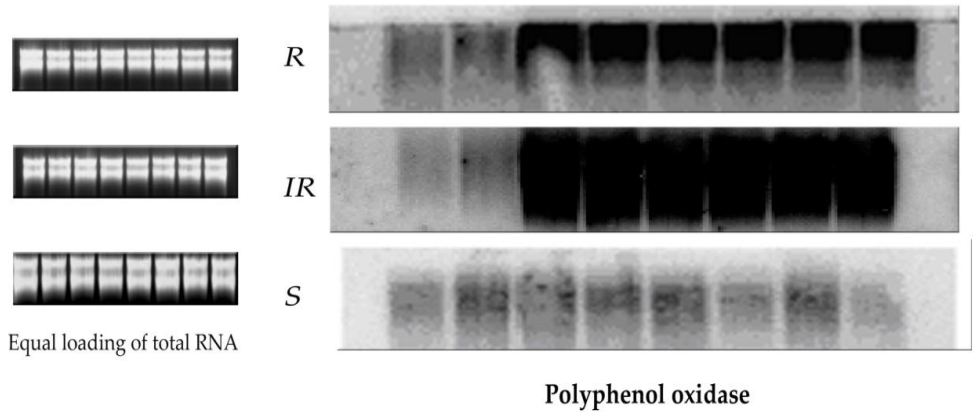

Figure 5: Northern blot analysis showing the temporal pattern of accumulation of polyphenol oxidase in resistant (R), induced resistant (IR – treated with *B. pumilus INR7*) and susceptible (S) pearl millet seedlings after inoculation with *S. graminicola*

Plants have unique responses to specific physical or chemical stimuli and this can be manifested by induction of genes putatively involved in a defense system. Induced systemic resistance (ISR) depends on the timely amassing of multiple transcripts and gene products, like hydrolytic enzymes and PR-proteins, which are known to stimulate elicitors from pathogen or host cell walls, that influence pathogens directly or indirectly by supporting the formation of structural barriers, or other biochemical reactions associated with plant defenses[11,12]. To understand plant defense responses to inducer treatments, it is important to study/characterize the expression/regulation of genes that encode various defense enzymes and signaling compounds. The molecular mechanisms underlying rhizobacteria-mediated ISR are to a large extent unknown. Peroxidase, lipoxygenase, and phenylalanine ammonia lyase are linked to the ISR pathway regulated by jasmonates and ethylene

and that is activated by saprophytic microorganisms including rhizobacteria[13] and therefore, studying the transcript accumulation pattern of these enzymes is necessary.

This study reports the transcript accumulation pattern of defense enzymes during PGPR mediated ISR in pearl millet downy mildew interaction. For most of the genes examined, the highest levels of transcripts were observed in resistant seedlings followed by induced resistant seedlings in comparison with the susceptible seedlings. For each gene, very low levels of RNA were present in susceptible seedlings. Inducer treatment also caused an increase in transcript accumulation, but generally not to the same degree as resistant seedlings. This was not the case for PAL and PPO where the highest transcript levels were observed in induced resistant seedlings. Measurement of mRNA accumulation demonstrates that genes encoding PAL and PPO were induced very prominently by PGPR treatment. Transcripts for each gene were also induced in susceptible seedlings, although not to the same level as in resistant and induced resistant seedlings. The PPO transcript was consistently the most strongly induced of all the genes examined. The enzyme products of the genes examined here are predicted to be involved in the biosynthesis of defense compounds, so it is not surprising that transcripts accumulated to high levels following pathogen inoculation.

β-1,3-glucanases and chitinases are frequently associated with a role in plant defense, and were first described as belonging to the pathogenesis-related family of proteins which accumulate rapidly in response to pathogen attack or stress. It is postulated that β -1,3-glucanases participate in the active defense response of plants to pathogens by releasing elicitors from fungal cell wall [14]. Chitinases include protein inhibitors of fungal hydrolases, and plant hydrolases which exert their hydrolytic activity towards fungal cell walls, causing lysis and/or release of elicitor-active fragments[15]. A role in plant defense mechanism has been assigned to β-1,3-glucanase and chitinase in a large number of species where pathogen induces earlier and higher increase in these enzyme activities in incompatible host-parasite interactions than in compatible ones[16-18].

The present study evidenced the accumulation of glucanase transcripts in all resistant, induced resistant and susceptible seedlings. However, the intensity was high in resistant and induced resistant seedlings compared to the susceptible seedlings. Maximum accumulation of glucanase was observed at 24 hpi in resistant seedlings, 48 hpi in induced resistant seedlings and 72 hpi in susceptible seedlings. At 24 hpi glucanase transcript accumulation was 2.7 folds higher than induced resistant seedlings. Our data demonstrate that in inoculated seedlings, chitinase activities were statistically highest in the resistant. The increased accumulation of these transcripts in the resistant and induced resistant seedlings may play both a direct protective role by degrading fungal cell wall component or an indirect role in the plant defense mechanism by releasing some elicitors from the decaying fungal cell wall that stimulate phytoalexin accumulation in the host plant. In fact, it is well known that β -1,3-glucanase, in combination with chitinase release oligosaccharide, signal molecules that can activate a variety of plant defence events[19,20].

The accumulation of PAL mRNA and activity of PAL enzyme vary greatly in response to various stress conditions and pathogen infection[21,22]. Several studies have shown a close correlation between induction of lignification and an increased synthesis of PAL enzyme following infection with the fungus and lignification is an important defense response in resistance. Therefore, PAL can be termed as an important defense enzyme and an indicator of PGPR mediated resistance. The results indicated the accumulation of PAL transcripts in all resistant, induced resistant and susceptible seedlings. However, the intensity was high in induced resistant and resistant seedlings compared to the susceptible seedlings. Maximum accumulation of PAL was observed at 48 hpi in induced resistant seedlings, 72 hpi in resistant seedlings and susceptible seedlings. At 48 hpi PAL transcript accumulation was 7.7 folds higher than induced resistant seedlings. Phenylalanine ammonia-lyase (PAL) plays a key role in linking primary metabolism to phenylpropanoid metabolism.

Peroxidases represent another component of an early response system in plants to pathogen attack. The products of these enzymes, in the presence of a suitable hydrogen donor and hydrogen peroxide can inactivate fungi, bacteria and viruses[23]. Both the timing and the localization of increased peroxidase activity may be important in limiting pathogen infections. In the current analysis accumulation of peroxidase transcripts were detected at all time intervals in resistant, induced resistant and susceptible seedlings. However, the intensity was higher in resistant and induced resistant seedlings compared to susceptible seedlings, further confirming the significance of this enzyme in plant defense.

Involvement of PPO during defense against plant pathogens is less studied, there are only two reports indicating the importance of PPO as a defense enzyme. Overexpression of polyphenol oxidase in transgenic tomato plants resulted in enhanced resistance to *Pseudomonas syringae* [24]. Mohammadi and Kazemi[25] showed the involvement of PPO in resistance against *Fusarium graminareum* during induced resistance. However, the involvement of PPO during induced resistance, particularly PGPR mediated induced resistance is not studied in any host-pathogen system. Further we state that PPO can be a putative marker of PGPR mediated ISR in pearl millet. PPO transcripts were present in the constitutive levels in all categories of seedlings. However, following pathogen inoculation, there was a marked difference in the transcript accumulation in resistant, induced resistant and susceptible seedlings with highest accumulation in induced resistant seedlings followed by resistant seedlings. At 24 hpi the transcript accumulation of PPO was 13.2 folds more in induced resistant seedlings compared to the control.

Our results are in line with earlier studies which have demonstrated that PGPR induced resistance against various plant pathogens in different crops is accompanied with significant changes in the pattern of expression of genes of various defense enzymes. Liang *et al*., [26] reported showed that the PGPR strain *Bacillus megaterium* L8 induced resistance in cucumber against seedling damping-off caused by *Pythium aphanidermatum* by increased expression of plant defense-related enzymes PAL, POX, PPO, superoxide dismutase and catalase. *Pseudomonas fluorescens* treatment to tomato seedlings protected against bacterial wilt disease caused by *Ralstonia solanacearum* by enhanced activities of PAL, POX, PPO and lipoxygenase (LOX) [27].

An endophytic rhizobacteria *Pseudomonas fluorescens* PICF7 triggered resistance against *Verticillium* wilt of olive by inducting higher expression of genes coding for LOX, PAL and catalase [28]. *Bacillus subtilis* CBR05 induced systemic resistance in tomato against soft rot disease caused by *Erwinia carotovora* subsp. *Carotovora* which was associated with significant increase in transcripts of the enzymes of glucanase and PAL [29]. A plant-growth–promoting rhizobacterium *Enterobacter asburiae* BQ9, induced resistance to Tomato yellow leaf curl virus (TYLCV) which correlated with enhanced expression of genes of PAL, POX, catalase, and superoxide dismutase[30]. Systemic resistance induced by *Bacillus amyloliquefaciens* NC6 derived protein elicitor (PeBA1) in tomato against a broad spectrum of pathogens including TMV and *Botrytis cinerea* was accompanied with upregulation of defence genes like PAL and PR proteins[31].

Conclusion

Plants develop a complex variety of events that involve synthesis and accumulation of new proteins that can have direct or indirect action during pathogenesis. The coordinated induction of several PR proteins that may act synergistically is part of the defense strategy that plants activate against the invading pathogen and may limit the colonization of the plant by inhibiting pathogen growth. Pearl millet seedling inoculation with *S. graminicola* was characterized in our experiments by an increase in β-1,3-glucanase, chitinase, PAL, POX, and PPO activity in the resistant and induced resistant seedlings. These alterations could be correlated to disease tolerance through the reinforcement of the lignification of the cell wall and other defense responses. The results from our experiments reveal that ISR by PGPR treatment can cause systemic gene responses as evidenced by accumulated transcripts for both for defense enzymes showing that gene-induction signals are being transported through the plant though the inducer and challenger remained spatially separated. In induced resistant seedlings, the highest levels of transcript accumulation were generally observed after pathogen inoculation. This result is indicative of the presence of specific elicitors or signal molecules triggered by PGPR treatment or a unique type of damage during host-pathogen interaction as compared to resistant seedlings. Understanding the regulation of the genes described here might aid

ultimately in manipulation of plant defense responses or in the selection of promising and potential PGPR strains and/or improvement of PGPR strains. The role of these genes' products in defense is emphasized by the strong and rapid induction of transcripts that occurred following PGPR treatment and pathogen inoculation. With the basic molecular characterization of PGPR mediated induced resistance reported here, targeted studies of the application of these genes in genetic engineering of PGPR strains and their use as markers of ISR can be carried out. The enzymes these genes encode, and the promoter sequences that control their regulation, might provide valuable tools in production of plants that are more pest-resistant.

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