RESEARCH ARTICLE

Transcript profiling of vital defense proteins upregulated during 3, 5-Dichloroanthranilic acid (DCA) mediated induced resistance against pearl millet downy mildew disease

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ABSTRACT

Downy mildew disease caused by the biotrophic oomycete *Sclerospora graminicola* is the main constraint for pearl millet production incurring huge yield and economic losses. The synthetic resistance elicitor 3, 5-Dichloroanthranilic acid (DCA) when applied exogenously as seed treatment to pearl millet at the concentration of 100 µM protected pearl millet plants by inducing systemic and durable resistance against downy mildew. This enhanced resistance correlated with the up regulation of various defense genes and proteins. Transcripts of mRNA of the defense enzymes PAL and POX were significantly enhanced many folds in comparison to the untreated control. The cell wall cross-linking protein HRGPs also showed significant overexpression in DCA treated seedlings compare to the control. Pathogenesis related proteins PR-1 and PR-5 which are regarded as markers of induced resistance were significantly over expressed in DCA treated seedlings, and expression of PR-5 was more than that of the resistant check. Early and increased expression of defense enzymes PAL and POX and defense proteins HRGPs, PR-1 and PR-5 are attributed as causes for enhanced DM protection that is offered by DCA treatment to pearl millet seeds. Changes in transcriptional profiles of defense enzyme sand proteins triggered by DCA clearly resemble typical defense-related responses elicited during elicitor induced resistance suggesting a potential for DCA in management of plant disease in general and pearl millet downy mildew disease in particular.

Keywords: Pearl millet downy mildew, induced resistance, defense genes, PR-proteins.

INTRODUCTION

Prior-treatment of host plants which are susceptible with suitable elicitors either biotic or abiotic will enhance the resistance of the plants, both at the site of pathogen attack as well as the other parts distant from

the site of pathogen invasion. This phenomenon is referred to as systemic acquired resistance (SAR) which is effective against a broad spectrum of plant pathogens and is long lasting (Ryals *et al*., 1996). Upstream to the development of systemic acquired resistance, several biochemical and molecular events takes place in the host plants particularly many defense related responses including the synthesis and accumulation of defense enzymes, phenolic compounds, pathogenesis-related proteins, reactive oxygen species, signaling molecules etc. (Ryals *et al*., 1996; Conrath *et al*., 2001).

Development of systemic acquired resistance is dependent on a coordinated expression of a set of complex SAR genes which majorly comprise of pathogenesis related (PR) proteins (Conrath *et al*., 2001). Though PR proteins are induced during plant tissues during normal growth and development, their induction and accumulation at higher levels during a pathogen infection is important for eliciting defense responses (Van Loon, 1999).

Therefore, the expression and accumulation of some of these PR proteins is regarded as a marker for identifying the systemic acquired resistance in plants. In several studies which have demonstrated induced resistance in crop plants against a wide range of plant pathogens there has been significant correlation between the resistance development and the expression of PR proteins. Salicylic acid (SA), beta-aminobutyric acid (BABA), Chitosan and INA were tested against *Alternaria* leaf blight in tomato plants and was found that all these elicitors induced systemic acquired resistance against the leaf blight pathogen which was associated with increase in the levels of the PR proteins chitinase and β1,3-glucanase (Raut and Borkar, 2014). BABA treatment potentiated defense responses in squash plants and induced resistance against powdery mildew disease and this phenomenon was associated with enhanced expression of genes of the PR-1 proteins and defense enzymes phenylalanine ammonia lyase (PAL) and peroxidase (POX) (Zeighaminejad *et al*., 2016). An elicitor protein NC6 isolated from *Bacillus amyloliquefaciens* elicited systemic resistance against different pathogens including tobacco mosaic virus and *Botrytis cinerea*, this resistance elicitation was due to enhanced expression of various defenses like hypersensitive reactions, enhanced accumulation of hydrogen-peroxide, overexpression o PR proteins like P1a, PR1b, PR5 and defense enzymes like PAL (Wang *et al*., 2016).

Pearl millet (*Pennisetum glaucum* [L.] R. Br.) is one of the most drought resistant grains in commercial production and is able to grow in areas that experience frequent periods of dry weather during the vegetative or reproductive phases. Pearl millet is tolerant of sandy and acidic soils, more efficient in utilization of soil moisture, has a higher level of heat tolerance making it more desirable in lower input, dry land agriculture production systems especially in the semi-arid regions. Pearl millet is the third most important cereal crop in India and is grown over 10 million hectares mainly as a rain fed crop with an annual production of 9.5 million tonnes (Yadav, 2016). However, downy mildew disease caused by the oomycete *Sclerospora graminicola* (Sacc.) J. Schröt is the major constraint for pearl millet production in India and causes yield losses ranging ranges form 20–40% annually (Thakur *et al*., (2011). In recent years, induction of resistance is being explored as an importnat management strategy and in our pervious study we have demonstrated that the synthetic elicitor 3, 5-Dichloroanthranilic acid (DCA) is highly promising as an elicitor for combating pearl millet downy mildew disease (Lavanya and Amruthesh, 2016). The aim of the present study was to elucidate the role and importance of certain PR proteins and defense enzymes at molecular level by analyzing the transcript accumulation profiles during DCA induced resistance against pearl millet downy mildew disease.

MATERIALS AND METHODS

Host

Seeds of pearl millet cultivars highly susceptible(7042S) and highly resistant (IP 18292) to the downy mildew pathogen were obtained from International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patencheru, Hyderabad, Andhra Pradesh, India, were used throughout the study.

Pathogen source and inoculum preparation

Downy mildew pathogen *S. graminicola* was isolated from susceptible cv.7042S. The susceptible pathogen was maintained on the same cultivar prior to use under greenhouse conditions (temperature of 22 ±2 ºC and relative humidity of 80 %). Pearl millet leaves showing prolific sporulation of *S. graminicola* on the

abaxial side were collected in the evening hours. The collected leaves were thoroughly washed under running tap water to remove dust and old sporangia. Then the collected leaves were blot dried and cut into smaller pieces, and placed in moist chamber for sporulation. The next morning, Fresh sporangia were collected and zoospores were released into sterile distilled water. Zoospore concentration was adjusted to 4 X 10^4/ml using hemocytometer and used as inoculum for all inoculation experiments (Safeeulla, 1976).

Preparation of inducer and seed treatment: DCA was obtained from Sigma. DCA solutions were prepared by dissolving 100 µM in DMSO (1µl/ml) of 100mL of sterile distilled water and kept for constant agitation for 2h for complete dissolution. For seed treatment, pearl millet seeds were surface sterilized with 0.02% mercuric chloride solution for 4-5 min and then thoroughly rinsed in sterile distilled water. Seeds were submerged in 10ml of inducer solution. Treated seeds were incubated at 25^0C in a rotary shaker for 4h to facilitate the seed treatment. For the same time interval, Seeds treated with sterile distilled water served as control.

Plating of treated seeds

7042S seed treatments with different elicitors were same as described earlier. In addition IP 18292 seeds treated with distilled water served as resistant check. After treatment, the seeds were plated on pre-soaked blotters in perspex plates and incubated for two days.

Challenge inoculation and harvesting of seedlings

Two-day-old seedlings were root-dip inoculated with a zoospore suspension of 40,000 zoospores/ml, and incubated in dark at 25 + 2°C. One set of the treated seedlings were inoculated with sterile distilled water which served as uninoculated control. A total of 1g seedlings for each experiment in three replicates were harvested at 0, 3, 6, 9, 12, 24, 48 and 72 h after inoculation (hai) and immediately wrapped in aluminum foil and stored at -80°C until further use.

Quantitative realtime PCR analysis (qPCR) for defense enzymes, hydroxyproline-rich glycoproteins, pathogenesis-related proteins.

a. RNA extraction

A total of 100 mg of frozen seedlings was ground to fine powder in a 2 ml SealRite microcentrifuge tube using stainless steel beads and an automated shaker SO-10M (Fluid Management, Wheeling, IL, USA). Total RNA was extracted from seedlings harvested at different times noted above by using the RNeasy plant mini Kit (Qiagen,) as per the manufacturer's instructions. Eluted RNA was stored at -80 °C and then treated with DNase I (RNase free) (Fermentas). The concentration and purity of RNA was determined by means of spectrophotometer and its integrity by agarose gel electrophoresis.

b. RT-PCR analysis

The relative quantitation of PAL (NM001174615.1), POX (EU492461), hydroxyproline-rich glycoproteins HRGP (GQ223398), PR1 (HQ699781.1), and PR5 (EU725133.1), *mRNAs* in pearl millet seedlings was done by using gene-specific primers, designed with Primer Express version 3.0 software (Applied Biosystems) (Table 1). PP2A (protein phosphatase 2A) served as endogenous reference gene. Primer specificities were confirmed by agarose gel electrophoresis of the RT-PCR products. Each qPCR reaction (20 µL) consisted of 1× SYBR Green PCR master mix (SYBR Green mix, Applied Biosystems), 3 pmol of each primer and 20 ng each of cDNA and used StepOnePlus™ Real-Time PCR Systems (Applied Biosystems). qPCR steps were: denaturation at 95°C for 10 min, 40 cycles of 15 s at 95°C, 60 s at 60°C.

Table 1. **Primer sequences used for qRT-PCR amplification**

Sl. No.	Target gene amplified	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')
1.	Phenyl alanine ammonia lyase	ATGGAGTGCGAGAACGGCC	CTGCGCGATGCTGAGGCT
2.	Peroxidase	CCCCAGAAGCACATTTGTGA	CATGGCTGCGGGCGGAG
3.	Hydorxy rich glyco proteins	GCCTAAGCCGAAGCCACCAA	GCGTGTAGGTCGGAGGAGTT
4.	PR1	TGGACGTGCCGCTGCCG	GAACTGCGCCGCCACACG
5.	PR-5	GCGTCCTCGGTCCTCCTG	CACACGCGGCCGGAGCTG
Reference housekeeping gene			
6.	Protein phosphatase 2A	TGAGAGCAGACAAATCACTCAA	AAGAGCTGTGAGAGGCAAATAA

At the end of each reaction, a melting curve was created using a single cycle consisting of 15 s at 95°C and 60 s at 60°C. This was followed by a slow temperature increase to 95°C at the rate of 0.3°C s^{-1}. The quantification of target mRNAs used a comparative Ct method (Livak and Schmittgen, 2001).

Data analysis

Data were analyzed separately for each experiment and were subjected to arcsine transformation and analysis of variance (JMP Software; SAS Institute Inc., Cary, NC). Significance effects of treatments were determined by the magnitude of the F value (P =0.05). Treatment means were separated by Tukey's honest significant difference test.

RESULTS

PAL gene expression

Constitutive levels of PAL transcripts were detected in all categories of seedlings and the expression levels were higher in resistant and induced resistant (DCA treated) seedlings compared to the susceptible controls. PAL gene expression gradually increased from 3 hours post inoculation (hai) and peaked at 6 hai and decreased thereafter in resistant and DCA treated seedlings, whereas in susceptible control seedlings PAL gene expression peaked at 9 hai (Fig. 1). Maximum level of PAL gene expression was recorded in resistant seedlings at all time points, and PAL expression in resistant seedlings at 6 hai was 4.59 folds higher than the control seedlings. PAL expression in DCA treated seedlings was 3.72 folds higher than the control seedlings. PAL gene expression in DCA treated seedlings was significantly higher than the control seedlings at all time points.

POX gene expression

Constitutive levels of POX transcripts were detected in all categories of seedlings and the expression levels were significantly higher in DCA treated seedlings compared to the resistant and susceptible controls. POX gene expression gradually increased from 3 hai and peaked at 9 hai and decreased thereafter in all categories of seedlings. Maximum level of POX gene expression was recorded in resistant seedlings form 3 to72 hai closely followed by DCA treated seedlings (Fig. 2). At 9 hai POX expression in resistant samples was on par with DCA treated seedlings which were 4.87 and 4.76 folds higher than the susceptible control.

HRGP gene expression

Constitutive levels of HRGP transcripts were detected in all categories of seedlings and the expression levels were significantly higher in resistant compared to the DCA treated and control seedlings. HRGP gene expression gradually increased from 3 hai and peaked at 9 hai in all categories of seedlings (Fig. 3). Maximum level of HRGP gene expression was recorded in resistant seedlings at all time points except 12 hai where DCA treated seedlings recorded maximum HRGP expression. At 9 hai, and HRGP expression in resistant and DCA treated seedlings were 2.11 and 1.37 folds higher than the control seedlings respectively.

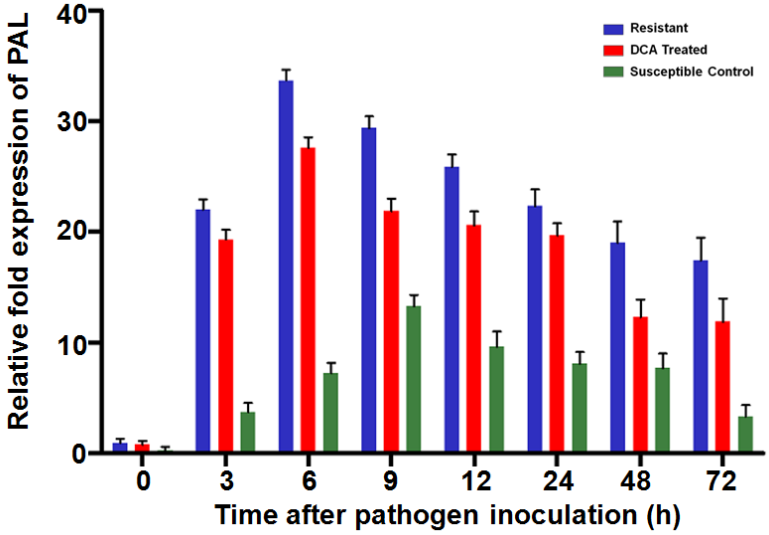

Figure 1: qRT-PCR determined relative expression of phenylalanine ammonia lyase (PAL) genes in two-day-old resistant (IP-18292), DCA treated (7042S treated with 100 µM DCA) and Susceptible control (untreated 7042S) pearl millet seedlings harvested 0, 3, 6, 9, 12, 24, 48, and 72 h after inoculation with *Sclerospora graminicola*

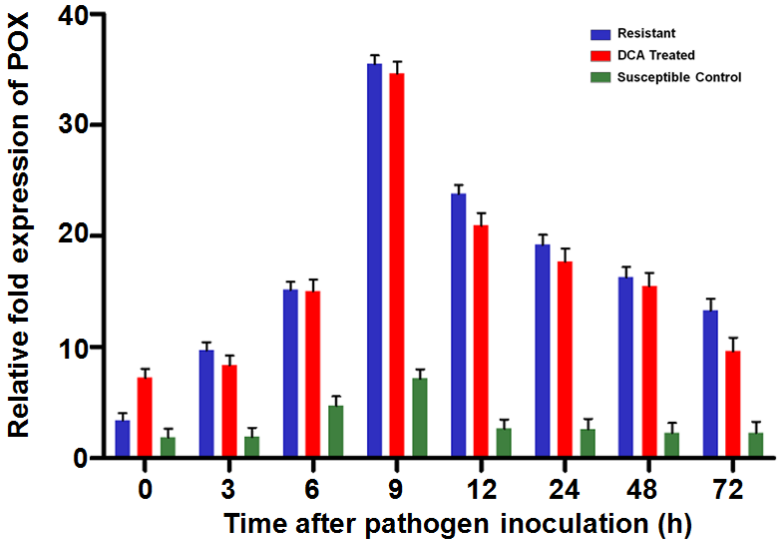

Figure 2: qRT-PCR determined relative expression of peroxidase (POX) genes in two-day-old resistant (IP-18292), DCA treated (7042S treated with 100 µM DCA) and Susceptible control (untreated 7042S) pearl millet seedlings harvested 0, 3, 6, 9, 12, 24, 48, and 72 h after inoculation with *Sclerospora graminicola*.

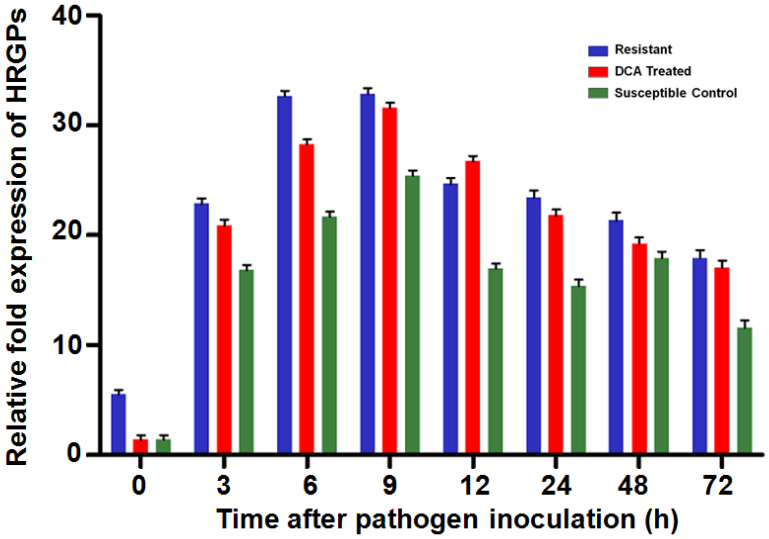

Figure 3: qRT-PCR determined relative expression of hydroxyproline-rich glycoproteins (HRGPs) genes in two-day-old resistant (IP-18292), DCA treated (7042S treated with 100 µM DCA) and Susceptible control (untreated 7042S) pearl millet seedlings harvested 0, 3, 6, 9, 12, 24, 48, and 72 h after inoculation with *Sclerospora graminicola*

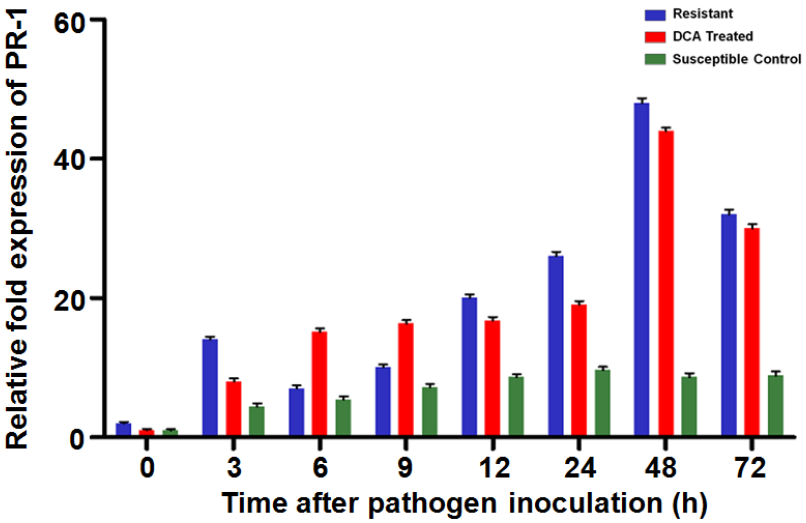

Figure 4: qRT-PCR determined relative expression of PR-1 genes in two-day-old resistant (IP-18292), DCA treated (7042S treated with 100 µM DCA) and Susceptible control (untreated 7042S) pearl millet seedlings harvested 0, 3, 6, 9, 12, 24, 48, and 72 h after inoculation with *Sclerospora graminicola*.

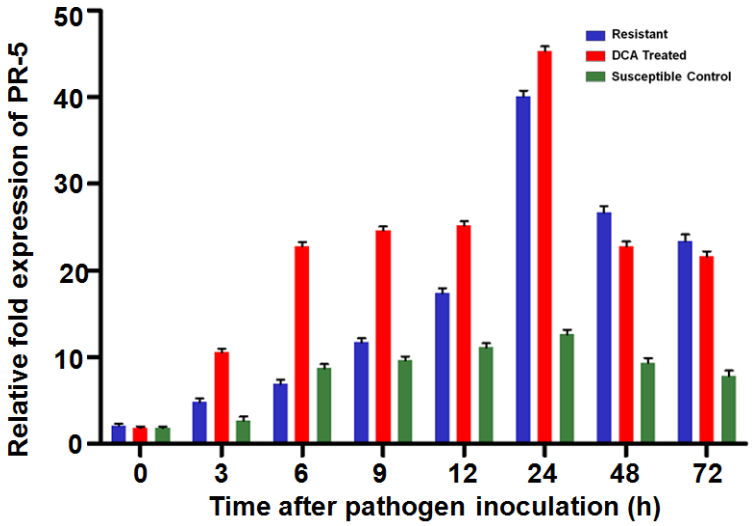

Figure 5: qRT-PCR determined relative expression of PR-5 genes in two-day-old resistant (IP-18292), DCA treated (7042S treated with 100 µM DCA) and Susceptible control (untreated 7042S) pearl millet seedlings harvested 0, 3, 6, 9, 12, 24, 48, and 72 h after inoculation with *Sclerospora graminicola*.

PR-1 gene expression

Constitutive levels of PR-1 transcripts were detected in all categories of seedlings. PR-1 gene expression gradually increased from 3hpi and peaked at 48 hai and decreased thereafter. PR-1 gene expression was maximum in resistant treated seedlings, except form 6 to 9 hai where DCA treated seedlings was significantly higher than both of resistant and susceptible seedlings. At 48 hai PR-1 expression in resistant and DCA treated seedlings was 5.61 and 5.29 folds higher than that of the susceptible control respectively (Fig. 4).

PR-5 gene expression

Constitutive levels of PR-5 transcripts were detected in all categories of seedlings. PR-5 gene expression gradually increased from 3 hai and peaked at 24 hai and decreased thereafter. Interestingly, up to 24 hai, maximum PR-5 activity was recorded by DCA treated seedlings which were even significantly higher than the resistant seedlings. At 24 hai PR-5 expression in DCA treated seedlings was 3.66 and 3.31 folds higher than that of the resistant and susceptible control respectively (Fig. 5).

DISCUSSION

It is established that during the process of systemic acquired resistance host defense mechanism gets primed and de novo expression of defense-related genes get upregulated leading to enhanced expression and de novo synthesis and accumulation of pathogenesis-related 'PR' proteins in uninfected tissues, thereby protecting them against any future pathogen attack (Ramos Solano *et al*., 2008). Though PR proteins and defense enzymes have a general role in plant defenses against pathogen infection, their accumulation is faster and higher in those plants which have been prior primed by certain elicitors which further get elevated following a pathogen inoculation.

The findings of the present study clearly demonstrated that during DCA induced resistance against pearl millet downy mildew disease several genes of the defense enzymes like, PAL, POX, and defense proteins like HRGPs and also pathogenesis related proteins like PR-1 and PR-5 are upregulated and the speed and intensity of their induction and overexpression positively correlated with the degree of resistance induced by DCA. In general DCA treated seedlings showed earlier and higher mRNA transcript accumulation of PAL, POX, HRGPs, PR-1 and PR-5 genes compared to the controls.

PAL participates in the synthesis of plant secondary antiviral substances, which can measure the ability of plant disease resistance and play an important role in plant disease resistance responses. PAL is an inducible enzyme that positively regulate SA-dependent defence signaling to combat microbial pathogens and it is known that PAL gene expression responds to a variety of environmental stresses, including pathogen infection and elicitor treatments (Kim and

Hwang, 2014). Peroxidases are important enzymes that play a key role in several metabolic responses induced during plant defence which include auxin metabolism, lignin and suberin formation, cross-linking of cell wall components, phytoalexin synthesis, and the metabolism of ROS and RNS. Peroxidases are a well-known class of PR proteins belonging PR-protein 9 subfamily whose gene expression in higher plants is known to be induced in host plant tissues by pathogen infection and elicitor treatments (Almagro *et al*., 2009).

The results of the present study showed that constitutive levels of PAL transcripts were higher in resistant and DCA treated seedlings compared to the susceptible controls. In DCA treated seedlings PAL gene expression was highest at 6 hai and was 3.72 folds higher than the susceptible control. Similarly, constitutive levels of POX transcripts were detected in all categories of seedlings. In DCA treated seedlings POX activity peaked at 9 hai and was 4.76 folds higher than that of the susceptible control. Our findings are in line with many other previous studies which have clearly demonstrated the role of PAL and POX gene expression during elicitor induced resistance against a wide range of pathogens in different crop plants. Bacterial canker of citrus caused by *Xanthomonas citri* subsp. *citri* was effectively controlled by the inducing resistance ability of L-arginine, L-methionine, L-ornithine, treatment which over expressed the transcripts of PAL and POX activities and heightened resistance against *Xanthomonas campestris* pv. *campestris* (Hasabi *et al*., 2014). In *Capsicum annuum* plants Thiamin (B1) and riboflavin (B2) induced systemic resistance against Tobacco mosaic virus infection and the levels of PAL and POX and some of the pathogenesis related proteins, PR4, PR9, andPR10 were overexpressed as indicated by RT-PCR studies (Torky, 2016).

Plant cell walls contain the structural proteins hydroxyproline-rich glycoproteins (HRGPs) in abundance and their involvement in plant defense is established both in monocots and dicots (Shailasree *et al.,* 2004; Mazau and Esquerre-Tugaye, 1986). HRGPs play a vital role in cell wall strengthening during an infection and they are known to primarily accumulate at the sites of attempted infection and also in within the papillae depositions (Brown *et al*., 1995). Crosslinkings of HRGPs with the other components of the cell wall is mediated by the generation and accumulation of H_2O_2 and the enzyme peroxidase and increased cross liking of HRGPs and other cell wall components which fortify the cell walls making it for pathogen digestion. (Iiyama *et al*., 1994). The results of the present study showed that constitutive levels of HRGP transcripts in all categories of seedlings with or without pathogen inoculation and the expression levels were significantly higher in resistant seedlings compared to the DCA treated and susceptible controls. DCA treated seedlings recorded 2.11 folds higher HRGPs expression in comparison to the untreated control. In bean plants, hybridizable HRGP mRNA accumulation was higher in elicitor treated plants in comparison to the untreated controls and this HRGPs enhancement led to development of resistance against *Colletotrichum lindemuthianum*, the causal agent of anthracnose in beans (Showalter *et al.,* 1985). Application of elicitors like SA and other fungi derived elicitors have shown higher induction and accumulation of HRGP mRNA in parsley cells (Thulke and Conrath, 1998).

Expression of certain PR genes is coincident with development of resistance, and the induction/activation of PR genes especially PR-1, PR-2 and PR-5 are considered as markers of systemic acquired resistance (Ward *et al*., 1991). PR-1 are commonly regarded as markers of systemic acquired resistance as they are the most dominant group of PR proteins expressed during induction of SAR (Sticher *et al*., 1997), however their exact functions are still not understood. The PR-5 family belongs to the thaumatin-like proteins with homology to permatins, that permeabilize fungal membranes and they have been shown to have antifungal activity, particularly against oomycetes. (Van Loon and Van Strien, 1999).

The results of the present study showed that, **c**onstitutive levels of PR-1 transcripts were detected in all categories of seedlings with or without pathogen inoculation and the expression levels were significantly higher in resistant and DCA treated seedlings compared to the other susceptible controls. PR-1 expression in DCA treated seedlings peaked at 48 hai which was 5.29 folds higher than that of the untreated control. Similarly**, c**onstitutive levels of PR-5 transcripts were detected in all categories of seedlings. However, it was interstign to note that The PR-5 gene expression in DCA treated seedlings was even higher than that of the resistant check at 24 hai. At 24 hai highest PR-5 gene expression in DCA treated seedlings

was 3.66 folds higher than that of the susceptible control.

There are numerous reports that have reported the role of PR proteins in plant defense particularly during induced systemic resistance. The biocontrol bacterium *Paenibacillus alvei* K165 protected *Arabidopsis thaliana* against *Verticillium dahlia* via induced systemic resistance by concomitant activation and increased transient accumulation of the PR-1, PR-2, and PR-5 genes (Tjamos *et al*., 2005). Abscisic acid treated tomato seedlings showed resistance to the early blight pathogen Alternaria solani and upregualtion of the mRNA transcripts of PR1 (Song *et al*., 2011). A genome-wide transcript microarray analysis of the β-aminobutyric acid (BABA) induced resistance against *Phytophthora infestans* in potato revealed the expression of over 5000 transcripts differentially expressed and accumulation of PR proteins (Bengtsson *et al*., 2014). Wang *et al*., 2016, reported that a protein elicitor PeBA1 isolated from *Bacillus amyloliquefaciens* NC6 induced systemic resistance in tobacco against a broad spectrum of pathogens, including Tobacco mosaic virus (TMV) and the fungal pathogen *Botrytis cinerea* and the RT-PCR studies revealed that elicitor treatment up regulated the salicylic acid (SA)-responsive PR1a, PR1b, PR5 genes

CONCLUSION

To our knowledge, this is the first report of the various defense enzymes/proteins and their transcript accumulation pattern during DCA mediated induction of resistance, in any host pathogen system in general and a monocot-oomycete interaction in particular. Various defense enzymes and signal molecules acting co-operatively may contribute to the development of an effective mechanical and chemical defense barrier in pearl millet plants against *S. graminicola* invasion. This hypothesis is substantiated by our findings showing that high levels of PAL, POX, HRGPs, PR-1, and PR-5 gene activities in DCA treated pearl millet seedlings which are correlated with high levels of resistance to downy mildew disease.

Conflicts of interest: The authors stated that no conflicts of interest.

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